Developmental and Comparative Immunology 34 (2010) 1219–1229

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Analysis of evolutionarily conserved innate immune components in coral links immunity and symbiosis

E. Charlotte E. Kvennefors[a,b,*], William Leggat[c,d], Caroline C. Kerr[a], Tracy D. Ainsworth[c], Ove Hoegh-Guldberg[a,b,1], Andrew C. Barnes[a,e]

[a] Centre for Marine Studies, The University of Queensland, St. Lucia, QLD 4072, Australia
[b] ARC Centre of Excellence for Coral Reef Studies, The University of Queensland, St. Lucia, QLD 4072, Australia
[c] ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, QLD 4811, Australia
[d] School of Pharmacy and Molecular Sciences, James Cook University, Townsville, QLD 4811, Australia
[e] School of Biological Sciences, The University of Queensland, St. Lucia, QLD 4072, Australia

ARTICLE INFO

Article history:
Received 24 May 2010
Received in revised form 23 June 2010
Accepted 23 June 2010
Available online 8 July 2010

Keywords:
Coral
Immunity
Symbiosis
Complement C3
Mannose binding lectin
Acropora millepora

ABSTRACT

Reef-building corals are representatives of one of the earliest diverging metazoan lineages and are experiencing increases in bleaching events (breakdown of the coral-*Symbiodinium* symbiosis) and disease outbreaks. The present study investigates the roles of two pattern recognition proteins, the mannose binding lectin Millectin and a complement factor C3-like protein (C3-Am), in the coral *Acropora millepora*. The results indicate that the innate immune functions of these molecules are conserved and arose early in evolution. C3-Am is expressed in response to injury, and may function as an opsonin. In contrast, Millectin expression is up-regulated in response to lipopolysaccharide and peptidoglycan. These observations, coupled with localization of Millectin in nematocysts in epidermal tissue, and reported binding of pathogens, are consistent with a key role for the lectin in innate immunity. Furthermore, Millectin was consistently detected binding to the symbiont *Symbiodinium in vivo*, indicating that the Millectin function of recognition and binding of non-self-entities may have been co-opted from an ancient innate immune system into a role in symbiosis.

1. Introduction

Reef-building corals are phylogenetically basal metazoans and are the major architects of coral reefs. The coral holobiont is an obligate association between the coral animal and its dinoflagellate endosymbiont, but this association is delicately balanced and coral reefs worldwide are increasingly threatened by mass coral bleaching events (breakdown of coral-dinoflagellate symbiosis) and outbreaks of poorly understood diseases (Harvell et al., 2002; Hughes et al., 2003; Ward and Lafferty, 2004). At present, very little is known about both the molecular processes that underpin the establishment and maintenance of the symbiosis, or coral immune responses to pathogens. Consequently, it is fundamental to determine the mechanisms involved in pathogen and symbiont recognition.

Corals have a simple body plan consisting of only two true tissue layers and no organs (Fig. 1). Depending on the stage of development (developing or adult coral) these tissues are referred to as the ectoderm/epithelium and the endoderm/gastroderm interlined by the mesoglea. The symbionts *Symbiodinium* reside mainly in gastrodermal areas located between gut and outer epithelium (barrier to external marine environment) in the oral end of the coral (indicated (a) in Fig. 1) and less frequently in gastrodermal areas between gut and skeleton (aboral areas) (Fig. 1b). Nematocysts are present in the epithelium in the oral end (Fig. 1a), but not in the calicoblastic epithelium (Fig. 1b). In the coral symbiosis, the eukaryote symbiont (*Symbiodinium*) is surrounded by a host-derived membrane (symbiosome) and contributes to the host's energy requirements through translocation of photosynthetically fixed carbon (Falkowski et al., 1984; Muscatine et al., 1984; Yellowlees et al., 2008). The majority of host corals only associate with specific genetic clades or sub-clades of *Symbiodinium* (e.g. (LaJeunesse, 2005)), suggesting the presence of a well-developed symbiont recognition system and leading to corals being portrayed as a model organism for symbiotic interactions (Weis et al., 2008). *Symbiodinium* can be either maternally inherited (vertical transmission) or obtained from the environment (horizontal transmission) through phagocytosis by coral tissues (Trench, 1987;

* Corresponding author at: Centre for Marine Studies, The University of Queensland, Research Road, Gehrman Building, Level 7, St. Lucia, QLD 4072, Australia. Tel.: +61 7 3365 4333; fax: +61 7 3365 4755.
E-mail address: c.kvennefors@uq.edu.au (E.C.E. Kvennefors).
[1] Present address: Global Change Institute, The University of Queensland, St. Lucia, QLD 4072, Australia.

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doi:10.1016/j.dci.2010.06.016

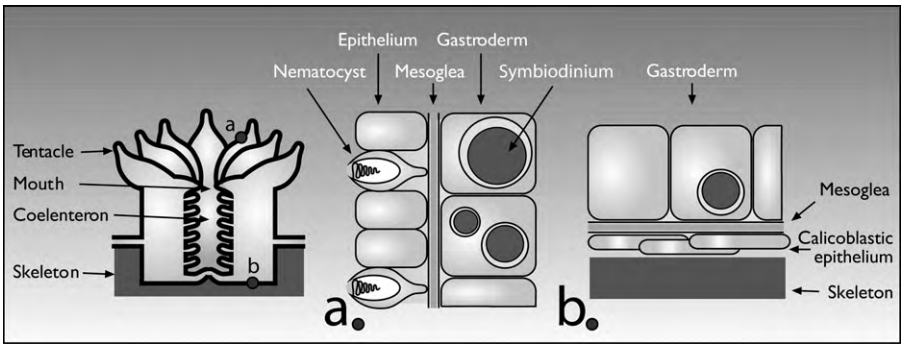

Fig. 1. Schematic representation of adult reef-building coral tissues and structures. Corals have only two true tissue layers, the ectoderm/epithelium and the endoderm/gastroderm interlined by the mesoglea, and no organs. *Symbiodinium* reside mainly in gastrodermal areas between gut and outer epithelium in the oral end of the coral (a) and less frequently in gastrodermal areas between gut and skeleton (aboral areas) (b). Nematocysts are present in the epithelium at the oral end (a), but not in the calicoblastic epithelium (b).

Schwarz et al., 1999; van Oppen, 2001; Coffroth et al., 2006; Marlow and Martindale, 2007). Those corals that obtain their symbionts horizontally may phagocytose several *Symbiodinium* strains along with food particles; however the resulting phagosome then avoids lysosome fusion by unknown means. Appropriate strains then multiply in phagosomes, establishing a stable symbiosis, whilst unsuitable strains are actively removed through cellular processes (Dunn and Weis, 2009). *Symbiodinium* is an alveolate (Zhang et al., 2005), as are many intracellular pathogens, suggesting that analogous mechanisms may underlie the utilization of host immune mechanisms by these eukaryotic pathogens and symbionts for entering the tissues. This hypothesis predicts that mechanisms of self/non-self-recognition have been co-opted into the symbiont establishment process (Weis et al., 2008; Kvennefors et al., 2008).

Recently it was shown that corals and other cnidarians contain a surprisingly high degree of genetic complexity (Kortschak et al., 2003; Miller et al., 2005; Putnam et al., 2007; Technau et al., 2005), and homologs of many proteins involved in immunity in vertebrates are present in cnidarians, suggesting ancient origins of these molecules in ancestral animals (Miller et al., 2007). The primary function of the innate immune system is to recognize specific patterns on non-self-entities (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002). These patterns are referred to as pathogen associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002), or microbe associated molecular patterns (MAMPs), and some of the best studied of these are microbial cell wall components such as lipopolysaccharide (LPS), peptidoglycan and mannan. These and other MAMPs are recognized by proteins referred to as pattern recognition receptors (PRRs) or pattern recognition proteins (PRPs) and this class of molecules includes both membrane bound proteins such as the Toll-like receptors and soluble proteins such as lectins. We recently characterised a mannose binding lectin from the coral *Acropora millepora* that is related to the vertebrate collectins, including mannose binding lectin (MBL) and surfactant protein A (SP-A), as well as to an ancestral ascidian MBL-like protein (Kvennefors et al., 2008). In vertebrates, SP-A can function as an opsonin for phagocytosis (Madan et al., 1997) and MBL is an important activator of many immune functions, including that of complement via the lectin-complement pathway (Takahashi et al., 2006). The coral lectin, known as Millectin, was shown to bind both bacteria and *Symbiodinium in vitro*, suggesting roles for the protein in both immunity and symbiont recruitment (Kvennefors et al., 2008). The detection of a high degree of sequence variation in the binding region of Millectin implies diversity in the binding repertoire (Kvennefors et al., 2008), perhaps to accommodate diverse target MAMPs.

The complement system is one arm of the vertebrate adaptive immune system, but complement factor C3 (C3) also participates in innate immunity via the opsonisation of foreign cells for phagocytosis and in inflammatory processes (Sahu and Lambris, 2001). The discovery of a vertebrate C3 homolog in the gorgonian coral *Swiftia exserta* (Dishaw et al., 2005) and similar molecules in other invertebrates (Raftos et al., 2002; Clow et al., 2004; Endo et al., 2006; Castillo et al., 2009) that predate the origins of the adaptive immune system suggests not only ancient origins for this molecule, but also for a prototype complement pathway. The C3 proteins belong to the thiolester-containing protein (TEP) family, which also includes opsonins from insects (Levashina et al., 2001), and sea urchin and horseshoe crab C3 with opsonic capabilities (Smith, 2002; Clow et al., 2004; Zhu et al., 2005; Ariki et al., 2008). In addition, the C3-like proteins from ascidians have been implicated in inflammatory responses (Raftos et al., 2003; Raftos et al., 2004). Interestingly, a 'primitive' form of the complement system, in which lectins interact with C3 for direct activation of phagocytosis, has been reported in invertebrates (Zhu et al., 2005; Endo et al., 2006). In ascidians, this opsonisation and activation includes the MBL-like protein (referred to as GBL; (Sekine et al., 2001)) related to Millectin. Therefore, further investigations to determine the roles in coral immunity of both Millectin and a recently discovered C3-like molecule from *A. millepora* (C3-Am) (Miller et al., 2007) are warranted.

The complex endosymbiosis between the coral host and *Symbiodinium* is likely to be regulated by non-self-recognition. Previously, many potential PRRs, including lectins, have been identified through microarray studies of corals (Miller et al., 2007; Schwarz et al., 2008), and lectins have been implicated in phagocytosis of *Symbiodinium* by the coral (Wood-Charlson et al., 2006). Investigations of PRPs in early immune pathways and their role in symbiosis will improve our understanding of the causes of symbiosis breakdown (bleaching) and disease outbreaks in these basal, but key, animals on coral reefs. Similarly, it will enhance our understanding of early evolutionary traits of innate immune mechanisms. The present study provides insight into the function of potential pattern recognition proteins, Millectin and C3-Am in immunity, symbiosis and host-microbe interactions in the coral model organism *A. millepora*.

2. Material and methods

2.1. Sequence alignment of A. millepora C3-like protein

The previously undescribed full cDNA sequence of a complement C3-like molecule (C3-Am; GenBank accession EF090257) derived from *Acropora millepora* EST analysis (full sequence provided by D. Miller; partial sequence described by Miller et al. (Miller et al., 2007)) were aligned and compared to previously characterised C3-related proteins by NCBI BLAST server. Translated