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Regulation of cnidarian–dinoflagellate mutualisms: Evidence that activation of a host TGFβ innate immune pathway promotes tolerance of the symbiont

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ABSTRACT

Animals must manage interactions with beneficial as well as detrimental microbes. Immunity therefore includes strategies for both resistance to and tolerance of microbial invaders. Transforming growth factor beta (TGFβ) cytokines have many functions in animals including a tolerance-promoting (tolerogenic) role in immunity in vertebrates. TGFβ pathways are present in basal metazoans such as cnidarians but their potential role in immunity has never been explored. This study takes a two-part approach to examining an immune function for TGFβ in cnidarians. First bioinformatic analyses of the model anemone *Aiptasia pallida* were used to identify TGFβ pathway components and explore the hypothesis that an immune function for TGFβs existed prior to the evolution of vertebrates. A TGFβ ligand from *A. pallida* was identified as one that groups closely with vertebrate TGFβs that have an immune function. Second, cellular analyses of *A. pallida* were used to examine a role for a TGFβ pathway in the regulation of cnidarian–dinoflagellate mutualisms. These interactions are stable under ambient conditions but collapse under elevated temperature, a phenomenon called cnidarian bleaching. Addition of exogenous human TGFβ suppressed an immune response measured as LPS-induced nitric oxide (NO) production by the host. Addition of anti-TGFβ to block a putative TGFβ pathway resulted in immune stimulation and a failure of the symbionts to successfully colonize the host. Finally, addition of exogenous TGFβ suppressed immune stimulation in heat-stressed animals and partially abolished a bleaching response. These findings suggest that the dinoflagellate symbionts somehow promote host tolerance through activation of tolerogenic host immune pathways, a strategy employed by some intracellular protozoan parasites during their invasion of vertebrates. Insight into the ancient, conserved nature of host–microbe interactions gained from this cnidarian–dinoflagellate model is valuable to understanding the evolution of immunity and its role in the regulation of both beneficial and detrimental associations.

1. Introduction

Transforming growth factor beta (TGFβ) cytokines are members of a large superfamily of signaling molecules, found throughout the Metazoa, that control a variety of cellular functions including developmental programming, tissue homeostasis and immunity (Massagué, 1998). TGFβ ligands group into several clades including: TGFβ *sensu stricto*, activins and bone morphogenetic proteins (BMPs), and all participate in highly conserved multi-component downstream signal transduction pathways (Fig. 1) (Heldin et al., 1997). Furthermore, there is crosstalk between different TGFβ ligands and different downstream pathways, greatly increasing the complexity of these pathways and resulting in functional versatility and cell-specific outcomes (Feng and Derynck, 2005; Herpin and Cunningham, 2007). Homologs to these pathway components have been identified in cnidarians where they have been implicated in several aspects of developmental programming but, to date, not in immunity (Hayward et al., 2002; Matus et al., 2006a,b; Saina and Technau, 2009; Samuel et al., 2001; Technau et al., 2005).

Functional characterization of TGFβs *sensu stricto* have been described only in vertebrates where they are involved in wide-ranging and largely anti-inflammatory mechanisms (Bogdan and Nathan, 1993; Hausmann et al., 1994; Ruscetti et al., 1993). Whether other TGFβ homologs play a role in immunity and self defense in invertebrates is an open question. A phylogenetic analysis of known TGFβs and putative TGFβ homologs from a variety of organisms identified a cluster of sequences with close phylogenetic

Abbreviations: BMP, bone morphogenetic protein; DAF-FM DA, 4-amino-5-methylamino2′,7′-difluorofluoroscein diacetate; DAN, 2,3-diaminonaphtalene; Dpp, decapentaplegic; DVR, decapentaplegic Vg-related; LPS, lipopolysaccharide; NO, nitric oxide; TGFβ, transforming growth factor beta.

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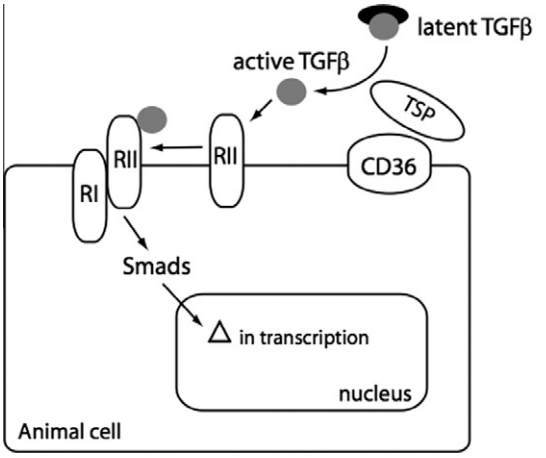

Fig. 1. A simplified TGFβ signalling pathway. TGFβ is secreted in a latent form (Li et al., 2006). The mechanisms of activation are not absolutely clear, but in many cases they involve interaction with extracellular matrix proteins including thrombospondin (TSP), specifically when TSP is bound to the scavenger receptor CD36 (Li et al., 2006; Masli et al., 2006; Yang et al., 2007). Once activated, TGFβ binds to a TGFβ receptor type II, a transmembrane protein. This complex in turn binds to a type I receptor to form a heterotetramer which proceeds to activate, by phosphorylation, a family of transcription factors called Smads (ten Dijke et al., 1996). Smads migrate to the nucleus and promote or inhibit transcription of a large variety of genes (Massague and Wotton, 2000).

placement to TGFβ *sensu stricto* that included three sequences from invertebrates (Herpin et al., 2004). This led Herpin and colleagues to suggest that these sequences were homologs of TGFβ *sensu stricto* and to hypothesize that a role for TGFβs in animal immunity existed prior to the divergence of these two groups. They therefore called for functional studies to explore this hypothesis. This has recently been verified in a report showing that TGFβ-like and BMP-like signals are anti-inflammatory in *Drosophila* (Clark et al., 2011). Another study identified two cDNA sequences closely related to the TGFβ *sensu stricto* group from the ctenophore (comb jelly) *Mnemiopsis* (Pang et al., 2011). Since ctenophores may be the earliest branching phylum on the animal tree of life (Dunn et al., 2008), it now seems likely that TGFβ *sensu stricto* ligands have existed since the origin of metazoans.

A key component of animal immunity is the management of host–microbe interactions both negative and beneficial (McFall-Ngai, 2008). To obtain a complete picture of the role of host immunity in host–microbe interactions, it is critical to consider both mechanisms of resistance – the ability to limit the burden of a microbial invader, and mechanisms of tolerance – the ability to limit the health impact of this burden (Schneider and Ayres, 2008). The relative contribution of these two processes could help determine the cost/benefit balance of an interaction, for example tipping a relationship over evolutionary time from a parasitic to a mutualistic one or *vice versa*. It is now well recognized that negative and beneficial interactions share many of the same host-microbe signalling pathways and cellular responses, including host innate immune responses to invading microbes (Hentschel et al., 2000; Relman, 2008; Schwarz, 2008). One tolerance-promoting (tolerogenic) mechanism employed by some parasites and pathogens of vertebrates involves modulation of the TGFβ pathway during invasion (Ndungu et al., 2005; Simmons et al., 2006; Waghabi et al., 2005). In this study, we explore the presence of a tolerogenic host innate immune response involving TGFβ in a cnidarian–dinoflagellate mutualism.

Cnidarian–dinoflagellate associations such as those that form reef-building corals are fundamentally important mutualistic symbioses in the marine environment. These partnerships provide the trophic and structural foundation of coral reef ecosystems (Dubinsky, 1990). This intracellular association is centered around nutrient exchange and is essential for both partners to thrive in nutrient-poor tropical seas. Cnidarian hosts, such as corals and anemones, harbor photosynthetic dinoflagellate endosymbionts, from the genus *Symbiodinium*, within gastrodermal cells in vacuoles of phagosomal origin known as symbiosomes. Initial colonization most often occurs when host gastrodermal cells lining the gastric cavity phagocitize symbionts ingested through the mouth during feeding (Colley and Trench, 1985; Davy et al., 2012).

Although cnidarian–dinoflagellate symbioses are stable in non-stressed conditions, various environmental stressors, most notably elevated temperature caused by global warming, can cause breakdown the partnership resulting in loss of symbionts from host tissues (Douglas, 2003). This phenomenon, known as coral bleaching, results in greatly reduced host fitness and can lead to reef destruction (Hoegh-Guldberg et al., 2007; van Oppen and Lough, 2009). The cellular mechanisms leading to symbiosis dysfunction and bleaching are largely unknown, but studies to date indicate that reactive oxygen and nitrogen species, host innate immunity, and host cell apoptosis all play a role (Detournay and Weis, 2011; Weis, 2008). Important to the present study are experiments that show elevated nitric oxide (NO) in host tissues of symbiotic anemones when they are subjected to a hyperthermic stress similar to levels that elicit a bleaching response (Detournay and Weis, 2011; Perez and Weis, 2006). Furthermore, addition of exogenous NO to anemones causes bleaching at ambient temperature. NO is a cytotoxic signaling molecule that plays a key role in the innate immune response in a variety of organisms (Fang, 2004). These results suggest that heat stress causes a cellular response related to an innate immune response that results in the elimination of the symbiont (Weis, 2008).

We were interested, therefore, in functional investigations of host innate immunity in cnidarian–dinoflagellate mutualisms. The goal was to provide evidence of TGFβ pathway components in a symbiotic cnidarian and evidence of their role in a tolerogenic immune mechanism that regulates these partnerships. We hypothesized that symbionts cause upregulation of a TGFβ and corresponding modulation of an immune response that results in persistence of symbionts in host tissues. Studies were performed in the model anemone *Aiptasia pallida* which harbors the same genus of dinoflagellate, *Symbiodinium* sp., as corals. Using the production of NO as a measure of immune activation, we show evidence of activation of a TGFβ pathway by the presence of symbionts, indicating that this pathway may have an immune function in cnidarians and may play a role in establishment and maintenance of a successful cnidarian–dinoflagellate symbiosis.

2. Materials and methods

2.1. Identification of TGFβ pathway components from A. pallida

TGFβ pathway components were identified in *A. pallida* through tBLASTn searches of AiptasiaBase, a publicly available *A. pallida* EST database consisting of ≈5000 unique sequences (http://aiptasia.cs.vassar.edu/AiptasiaBase/index.php) (Sunagawa et al., 2009) using human TGFβ pathway sequences as queries (see Table 1 for query sequences). An additional data set of 58,018 *A. pallida* contigs (Lehnert et al., 2012) was also searched in the same manner. Resulting *A. pallida* EST sequences were used as query sequences in a reciprocal BLASTx search of GenBank. The *A. pallida* TGFβ ligand sequence has been deposited in GenBank (GenBank ID: JX113692).

The alignment provided in the recent paper by Pang et al. (2011) formed the basis of a phylogenetic analysis of TGFβ ligands. The alignment was pruned to remove extraneous sequences,