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Thioester-containing proteins of the tick *Ixodes ricinus*: Gene expression, response to microbial challenge and their role in phagocytosis of the yeast *Candida albicans*

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ABSTRACT

The ability of ticks to act as vectors for a wide range of serious human and animal infectious diseases is apparently linked to the insufficiency of the tick immune system to effectively eliminate pathogens they transmit. At the tick-pathogen interface, an important role is presumably played by components of an ancient complement system that includes a repertoire of thioester-containing proteins (TEPs), which in *Ixodes sp.* comprises three α_2-macroglobulins (A_2M), three C3 complement component-related molecules (C3), two macroglobulin complement-related (Mcr) and one insect-type TEPs (Tep). In order to assess the function of TEPs in tick immunity, a quantitative real-time PCR expression analysis of tick TEPs was performed at various developmental stages *of Ixodes ricinus*, and in tissues dissected from adult females. Expression of *TEP* genes was mostly tissue specific; *IrA$_2$M1, IrC3-1, IrC3-3* were found to be expressed in cells of tick fat body adjacent to the tracheal trunks, *IrA$_2$M2* in hemocytes, *IrTep* in ovaries, *IrMcr1* in salivary glands and only *IrA$_2$M3, IrC3-2* and *IrMcr2* mRNAs were present in multiple organs. Expression of tick TEPs was further examined in response to injection of model microbes representing Gram-negative, Gram-positive bacteria and yeast. The greatest expression induction was observed for *IrA$_2$M1* and *IrC3-1* after challenge with the yeast *Candida albicans*. Phagocytosis of the yeast was strongly dependent on an active thioester bond and the subsequent silencing of individual tick TEPs by RNA interference demonstrated the involvement of IrC3-1 and IrMcr2. This result suggests the existence of a distinct complement-like pathway, different from that leading to phagocytosis of Gram-negative bacteria. Understanding of the tick immune response against model microbes should provide new concepts for investigating interactions between ticks and relevant tick-borne pathogens.

1. Introduction

Ticks are blood-sucking chelicerates capable of transmitting a large variety of viral, bacterial, fungal and protozoan pathogens, causing serious diseases of their vertebrate hosts, including humans (de la Fuente et al., 2008). The versatility of ticks as disease vectors can, in part, be attributed to the inability of their innate immune system to eliminate the pathogen during its acquisition, persistence or transmission (Hajdusek et al., 2013). Research on tick innate immunity during the last decade has revealed that ticks produce antimicrobial peptides, either in common with other arthropods (e.g. defensins, lysozymes), or tick-specific (e.g. microplusins) that are secreted to the gut lumen, hemolymph or saliva (for review see Kopacek et al., 2010; Sonenshine and Hynes, 2008). In addition, several components, presumably playing a role in a primordial complement system of ticks, such as fibrinogen-related lectins, α_2-macroglobulins, C3-complement related molecules and putative convertases have been identified and described (Buresova et al., 2009, 2011; Kopacek et al., 2012; Rego et al., 2006; Urbanova et al., 2014). The complexity of the tick immune response was studied in the American dog tick, *Dermacentor variabilis*, infected with a wide range of bacterial and fungal microbes including the important tick-borne pathogen *Anaplasma marginale* (Jaworski et al., 2010). Using the 454 sequencing platform, more than 30 genes putatively involved in tick immunity were identified, including genes that code for serpins, fibrinogen-related proteins, transglutaminase, calreticulin, superoxide dismutase,

Abbreviations: GFP, green fluorescent protein; IrA$_2$M1, 2, 3, *Ixodes ricinus* α_2-macroglobulin 1, 2, 3, respectively; IrC3-1, -2, -3, *I. ricinus* C3- complement component 1, 2, 3, respectively; IrMcr1, 2, *I. ricinus* macroglobulin complement related 1,2, respectively; IrTep, *I. ricinus* thioester-containing protein; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference; TEP, thioester-containing protein.

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galectin, defensins and thioester-containing proteins (Jaworski et al., 2010).

The family of thioester-containing proteins (TEPs), formerly referred to as proteins of the α_2-macroglobulin family, are defined, in most cases, by a highly reactive β-cysteinyl-γ-glutamyl thioester (TE) bond within a thioester domain. Among invertebrates, four phylogenetically distinct groups of TEPs can be recognized: (i) α_2-macroglobulin pan-protease inhibitors (A_2M); (ii) C3 complement component-related molecules (C3), characterized by the presence of an anaphylatoxin and a C-terminal netrin-like (NTR/complement C3/C4/C5) domain, (iii) insect-type TEPs (iTEPs), and (iv) molecules named macroglobulin complement-related (Mcrs) harboring an additional low density lipoprotein receptor class A (LDLa) domain (Buresova et al., 2011). To this end, only ticks have been reported to maintain all four invertebrate TEP groups during their evolution from the common ancestor of Arthropoda (Buresova et al., 2011). The genome of *Ixodes scapularis* contains genes encoding three α_2-macroglobulins (A_2M1, 2, 3), three C3-complement components (C3-1, -2, -3), two Mcrs (Mcr1, 2) and one insect-type TEP (Tep) (Buresova et al., 2011). Characterized tick α_2-macroglobulins, namely TAM from the soft tick *Ornithodoros moubata* (Kopacek et al., 2000; Saravanan et al., 2003) and IrAM from the hard tick *Ixodes ricinus* (here referred to as IrA_2M1) (Buresova et al., 2009) display a post-translational processing into two C-terminal (α) and N-terminal (β) chains held together by intrachain disulfide bridges, a feature typical for C3 and C4 complement components. Two putative α_2-macroglobulins, tagged as DvMrG1 and DvMrG2, and one C3-related molecule were identified among *D. variabilis* immune responsive genes (Jaworski et al., 2010). The spider *Hasarius adansoni* was reported to possess two genes encoding C3-type, one A_2M and one iTEP molecules (Sekiguchi et al., 2012). The A_2M present in the hemolymph of the horseshoe crab was isolated and characterized two decades ago (Iwaki et al., 1996; Quigley and Armstrong, 1985) and more recently, molecules related to the C3-complement component have been described to function in the primordial complement system of these living fossils (Ariki et al., 2008; Zhu et al., 2005). In the freshwater crayfish *Pacifastacus leniusculus*, two A_2Ms, one iTEP but no C3-related molecules were identified (Wu et al., 2012). The C3 group seems to have been completely lost during evolution of the Hexapodan lineages since no C3-related genes have been identified within any insect genome (Sekiguchi et al., 2012). Some insect (honey bee, ant, wasp, flour beetle) genomes contain genes phylogenetically closely related to the A_2Ms (Sekiguchi et al., 2012; Wu et al., 2012), although, to our knowledge, no functional A_2M protease inhibitors have been yet described from any insect species. The TEP family seems to be the most reduced in the dipteran insects, since they possess only the groups of iTEPs and Mcrs. The TEP family in *Drosophila melanogaster* comprises five genes coding for *DmTep1-5* (Lagueux et al., 2000), out of which the *DmTep5* is likely a pseudogene, and one *Mcr* (originally termed as *DmTep6*). The genome of the malaria mosquito, *Anopheles gambiae*, contains 15 genes encoding TEPs (AgTeps) (Christophides et al., 2002) where AgTep13 most likely represents the Mcr group (Bou Aoun et al., 2011; Buresova et al., 2011).

The repertoire of TEP proteins in arthropods is most likely to be arranged in several complement-like pathways, effective in specific defenses against different kinds of microbes (Levashina et al., 2001; Moita et al., 2005; Stroschein-Stevenson et al., 2006). In *A. gambiae*, the *AgTep1* gene encodes a mosquito opsonin that is involved in the killing of *Plasmodium berghei* ookinetes and thereby functions as a determinant of the mosquito's competence to vector malaria (Blandin et al., 2004; Levashina et al., 2001). RNAi silencing of *AgTep1* also significantly inhibits phagocytosis of *Escherichia coli* as well as the Gram-positive bacterium *Staphylococcus aureus* (Moita et al., 2005). A similar effect on phagocytosis of both bacteria was obtained upon RNAi silencing of *AgTep4*, whereas *AgTep3* depletion by RNAi only reduced phagocytosis of *E. coli* but not *S. aureus* (Moita et al., 2005). The fruit fly *DmTep2, DmTep3* and *DmMcr* (*DmTep6*) are specifically required for efficient phagocytosis of *E. coli, S. aureus* and *Candida albicans*, respectively, by cultured *Drosophila* S2 cells (Stroschein-Stevenson et al., 2006). *Drosophila* TEPs were further shown to act redundantly against different pathogens and their expression is not strictly required in the body cavity to protect the fruit fly against bacterial or fungal infection (Bou Aoun et al., 2011). In another study, *DmTep3* was reported to be involved in the defense of *Drosophila* larvae against entomopathogenic nematodes (Arefin et al., 2013). An Mcr molecule from the mosquito *Aedes aegypti* was recently reported to control dengue virus infection via expression of antimicrobial peptides exerting potent anti-viral activity (Xiao et al., 2014). Besides a role in immunity, a novel developmental function for DmMcr was recently discovered by two separate laboratories, demonstrating that the molecule was involved in an assembly of septate junctions and a formation of epithelial barriers (Batz et al., 2014; Hall et al., 2014).

The feasibility of RNA interference (RNAi) in ticks allowed us to exploit functional genomics to investigate the roles of all nine members of the tick TEP family (referred to as IrTEPs) in *I. ricinus*, the European vector of Lyme disease and tick-borne encephalitis virus (Buresova et al., 2011). Previously, we have shown that phagocytosis of the Gram-negative bacteria *Chryseobacterium indologenes* and *E. coli* by *I. ricinus* hemocytes depends on the same central molecule IrC3-3, possibly shared by two distinct pathways that involve IrA_2M1 or IrTep, respectively (Buresova et al., 2009, 2011). Here, we further contribute to the assessment of IrTEPs function in *I. ricinus* immunity and/or development by quantitative stage and tissue expression profiling and an analysis of the immune response upon injection of microbial models. Since the strongest immune response was observed against *C. albicans*, we further investigated which IrTEPs are involved in the phagocytosis of the yeast. Results indicate the existence in ticks of a specific complement-like response against fungi, comprising mainly IrC3-1 and IrMcr2, which is distinct from pathways leading to the phagocytosis of Gram-negative bacteria.

2. Materials and methods

2.1. Biological material

Adult females and males of *I. ricinus* were collected in České Budějovice in Czech Republic. All developmental stages (eggs, larvae, nymphs and adults) were maintained in wet chambers with a humidity of about 95%, temperature 24 °C and day/night period set to 15/9 h. Females were naturally fed in the presence of males on laboratory guinea pigs. The larvae were fed on guinea pigs, allowed to molt to nymphs and after 4–6 weeks, further fed on guinea pigs or rabbits. The molted adult females (pathogen free) were used for experiments described below. All laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 137/2008.

2.2. Tissue and developmental stage IrTEPs expression profiling by quantitative real-time PCR

Total RNA was isolated from eggs, larvae, nymphs and adult tick homogenates, and dissected tissues or hemolymph collected from semi-engorged *I. ricinus* females, as described previously (Urbanova et al., 2014). The resulting cDNA preparations from developmental stages and tissues were made in independent triplicates and served as templates for the following quantitative expression analyses by quantitative real-time PCR (qPCR) using the LightCycler 480 (Roche) and SYBR green chemistry. For reaction conditions and sequences of qPCR-*IrTEPs* forward and reverse primers, see