University of South Bohemia
Faculty of Science
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Ph.D. thesis

Function of the α_2-macroglobulin protein family in the immune response of the tick *Ixodes ricinus*

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České Budějovice, 2009

Burešová V., 2009: Function of the α_2-macroglobulin protein family in the immune response of the tick *Ixodes ricinus*. Ph.D. Thesis (in English) -77 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation:
The thioester containing proteins of α_2-macroglobulin family are evolutionary ancient components of innate immunity. We characterized IrAM – the protease inhibitor of α_2-macroglobulin class from the hard tick *Ixodes ricinus*, a vector of Lyme disease and tick-borne encephalitis. The functional study using RNA interference revealed that IrAM is involved in the phagocytosis of pathogenic *Chryseobacterium indologenes.* This is the first report demonstrating the link of the α_2-macroglobulin with cellular immune response. Data mining in the genome of a closely related *Ixodes scapularis* disclosed at least nine thioester proteins related to α_2-macroglobulin, C3 complement component and insect thioester proteins. Several orthologs have been already identified in *I. ricinus* providing a base for further RNA interference-based studies on the complement-like reactions at the tick-pathogen interface.

Financial Support: This work was supported by grants to P.K.: No. IAA6022307 and IAA600220603 from the Grant Agency of the Academy of Sciences of the Czech Republic, No. 206/06/0865 from the Grant Agency of the Czech Republic and the Research Centre No. LC06009.

I hereby declare that all the work summarized in this thesis was performed on my own or in collaboration with co-authors of the presented papers and with the help of the cited literature.

Veronika Burešová

České Budějovice, March 2009

ACKNOWLEDGMENT

I would like to thank kindly

- My supervisor Dr. Petr Kopáček for the scientific guidance,a numerous pieces of advice and never-ending encouragement during my study and work in laboratory

- Ondra, Zdeny, Dan for their help, precise work and for their friendships. It has been fun working with you

- Also, I thank other members and friends of our lab namely Helča, Lenka, Petr, Honza and Jindra for enjoyable lab atmosphere and all my friends from inside and outside the Institute of Parasitology for their friendship

- Prof. Libor Grubhoffer and prof. Tomáš Scholz for the opportunity to work at the Institute of Parasitology

- Last but not least, I want to thank my parents and Jirka for their support and love they gave me throughout many years of my studying

ABBREVIATIONS

α_2M	α_2-macroglobulin
aTEP	*Anopheles gambiae* thioester containing protein
DAPI	4,6-diamidino-2-fenylindol
dMcr	*Drosophila* macroglobulin complement related protein
dTEP	*Drosophila melanogaster* thioester containing protein
F-α_2M	α_2-macroglobulin family
GLY	L-glycine
GFP	Green fluorescent protein
Gram(-)	Gram-negative bacterium
Gram(+)	Gram-positive bacterium
IrAM-KD	Knockdown of IrAM
IrAM	*Ixodes ricinus* α_2-macroglobulin
IsAM	*Ixodes scapularis* α_2-macroglobulin
KD	Knockdown
MA	Methylamine
RNAi	RNA interference
RT-PCR	Reverse transcription-polymerase chain reaction
TEPs	Thioester containing proteins
TAM	*Ornithodoros moubata* α_2-macroglobulin

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INTRODUCTION

1. The innate immune system of invertebrates

The immune system of invertebrates plays a crucial role in the defense of organisms against pathogens. It is activated when physical barriers (cuticule, perithrophic matrix of midgut, or chitin layer in tracheal system) are overcome by an invading microorganism. The immune response of invertebrates has to rely on innate immunity, in contrast to vertebrates that also possess an adaptive immunity. Vertebrate adaptive immunity is based on lymphocytes (effector cells) with antigen specific receptors and the antibodies they produce. Essentially, they can recognize an unlimited number of various targets and are fundamental elements of immunological memory.

Innate immunity consists of humoral factors and cellular defense which are tightly linked. The invertebrate humoral immunity comprises antimicrobial peptides, coagulation factors, pro-phenoloxidase system, lectins, proteases inhibitors, and pattern recognition receptors. The cellular immune response is mainly represented by phagocytosis, encapsulation, or nodulation of invading microorganisms (for general schema see Figure 1).

The innate immune machinery is triggered upon pathogen recognition by pattern recognition receptors (PRRs). These receptors recognize pathogens associated molecular patterns (PAMPs) which are antigenic structures produced only by microbial pathogens exposed on their surface. The best known examples of PAMPs include: lipopolysaccharide (LPS) characteristic for Gram(-) bacteria, peptidoglycans of Gram(+) bacteria and β-1,3-glucans of fungi (Medzhitov and Janeway, 2000). PRRs are responsible for the activation of appropriate defense pathway and are expressed on the cell surface or secreted into the body fluids (Medzhitov and Janeway 2002). PRRs have been well described as activators of *Drosophila* Toll or Imd pathways. The activation results in the production of antimicrobial peptides (AMPs) (Lehane et al., 2004; Tzou et al., 2002). AMPs are small cationic peptides, predominantly membrane active which possess a prominent function in a defense against invading microorganisms (Hoffmann, 2003). They are synthesized by fat body or hemocytes and are secreted into the hemolymph (Leclerc and Reichhart, 2004). Some of them are conserved among many species (Bulet et al., 1999).

Recognition of PAMPs by PRRs or tissue damage can activate pro-phenoloxidase activating system (proPPO) (Soderhall et al., 1990; Kurata, 2006). The proPPO system is a part of some arthropods immune mechanisms, especially well described in insects and crustaceans. All known pro-phenoloxidases of arthropods are secreted as inactive precursors which are activated by the cascade of protease-activating enzymes. PPO system functions in wound healing, elimination of pathogens, and is associated with stimulating factors of a cellular defense (Soderhall et al., 1994; Cerenius et al., 2008). The ultimate result of proPPO cascade is melanization of pathogens or damaged tissues and also a production of cytotoxic reactive oxygen species (Cerenius and Soderhall, 2004).

Since invertebrates have an open circulatory system, the coagulation of hemolymph is a reaction protecting them from the loss of the body fluid and the dissemination of invading microbes after a injury (Theopold et al., 2002). The coagulation cascade was described in detail for the horseshoe crab. It is activated after PAMPs recognition and leads to the conversion of coagulogen (analog of mammalian fibrinogen) to an insoluble coagulin (fibrin) gel (Iwanaga and Lee, 2005). The activation of coagulation proteins also leads to the release of small cleavage products which act as antimicrobial peptides (Theopold et al., 2004).

An important role in innate immunity is played by sugar binding proteins-lectins, because a protein-sugar interaction is involved in many of immune responses in invertebrates especially in self/nonself recognition, opsonization, phagocytosis and defense against microbes. The best characterized lectins are from the horseshoe crab *Tachypleus tridentatus*, where Tachylectin 5A and 5B were shown to agglutinate bacteria (for rev. see Iwanaga, 2002).

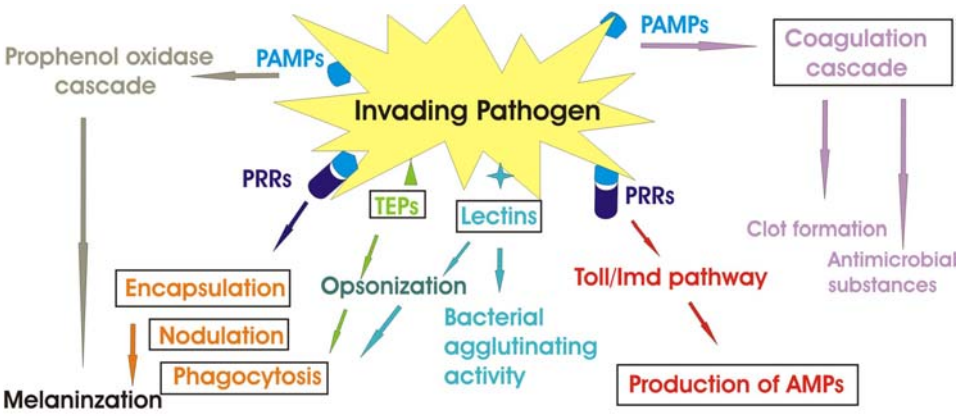


Figure 1: Invertebrate immune responses. Immune reactions present in ticks are in rectangles.

Hemocytes are the effector cells of a cellular immune response. Their main role is the production of many humoral molecules, protection against invading pathogens, and wound healing (Lavine and Strand, 2002). An amount of circulating hemocytes is obviously increased after a microbial infection. Different types of hemocytes are described in different invertebrate species. The most common hemocytes are granulocytes, plasmatocytes, spherule cells, oenocytoids and prohemocytes that are regarded as stem cells (Lavine and Strand, 2002; Jiravanichpaisal et al., 2006).

Cellular immunity involves phagocytosis, nodulation and encapsulation. Phagocytosis is an evolutionary conserved process serving for the removal of invading pathogens (Ratcliffe and Rowley, 1979). It could be hypothesized that invertebrate phagocytosis is similar with the mammalian one as well as a receptor-mediated defense response. When foreign particle (microbe) has been recognized by a specific receptor, a phagocytic process is initiated (Lavine and Strand, 2002; Marmaras and Lampropoulou, 2009). The next steps are a cytoskeleton modification, an intracellular transport, and the fusion of foreign particle in phagosome with lysosomes which form phagolysozome together. Then the target is destructed by toxic compounds like reactive oxygen species (ROS) and nitrogen compounds (Jiravanichpaisal et al., 2006; Pearson et al., 2003).

Nodulation results in the formation of hemocyte sheaths that surrounds invading bacteria (Ratcliffe and Gagen; 1977, Lavine and Strand, 2002). Encapsulation is a process very similar to nodulation, but in this reaction hemocytes surround much bigger objects such as parasitoids and nematodes. Multicellular capsule is made by hemocytes. Nodulation and encapsulation could be joined with the activation of the PPO system and the melanin production which is important for the destruction of parasites (Jiravanichpaisal et al., 2006).

2. Proteins of α_2-macroglobulin family – the ancient part of innate immunity

The proteins of α_2-macroglobulin family (F-α_2M), more generally named as thioester-containing proteins, have been found throughout the whole animal kingdom as the important components of humoral and cellular immunity (Blandin and Levashina, 2004). The proteins of F-α_2M comprise three main groups: i) C3/C4/C5 components of the complement system; ii) The insect thioester-containing proteins (TEPs); iii) universal

protease inhibitors of the α_2-macroglobulin class (α_2M). The presence of internal β-cysteinyl-γ-glutamyl thioester bond within the conserved motif GCGEQ is the hallmark for the most of F-α_2M members (Armstrong, 2006). These large proteins are secreted in the form of ~1500 amino acids long precursors which become functional upon a proteolytic activation. The activation of complement components is driven by a specific protease complex (convertase) which is followed by a conformational change and the exposure of the reactive thioester bond. Alpha$_2$-Ms are activated by a wide spectrum of attacking proteases and the proteolytic action is also assumed for the activation of some TEPs (Blandin and Levashina, 2004; Blandin et al., 2008).

2.1. The complement system

The complement of vertebrates consists of about 30 serum and membrane proteins that provide a variety of immune reactions (Figure 2). The major functions of complement are an inflammatory response mediated by anaphylatoxins (C3a, C5a), the opsonization of microbes (C3b), the promotion phagocytosis, and the formation of membrane-attack complex (C5b) followed by lysis of pathogens. Moreover, complement component C3b binds to B cell receptor that triggers antibodies production. There are three ways of complement activation in mammals: The lectin pathway where glycans (especially mannose residues) present on the surface of pathogens are recognized by the mannose-binding lectin (MBL) or ficollins. The classical pathway is activated by the binding of C1q components to the complex of antibodies and antigens or when C1q bounds directly to the microbial membrane. In alternative pathway, the complement can be activated directly by the presence of microbial cell surface. Generally, the activation of different components of complement by all these ways leads to the initiation of C3-convertase, which cleaves C3. It is a crucial event in the cascade resulting in the generation of effector molecules. C3b, that originates from C3 component after its cleavage, is important either for the induction of phagocytosis or it can promote the cleavage of the next component of complement - C5. C5a is the major mediator of an inflammatory response, while C5b together with C6, 7, 8 and 9 are responsible for the formation of membrane-attack complex (MAC) (DeFranco et al., 2007).

C3, the central component of mammalian complement, is an evolutionary ancient molecule. It has also been identified in jawless vertebrates, lamprey, hagfish, amphioxus, sea urchin and ascidian (Nonaka et al., 1984; Fujii et al., 1992; Suzuki et al., 2002; Smith

et al., 2001; Al-Sharif et al., 1998). The functional homologue of vertebrate C3 was as well identified in the "living fossils" – the horseshoe crabs *Carcinoscorpius rotundicauda* (CrC3) and *T. tridentatus* (TtC3). Both molecules were capable of binding to Gram(-) and Gram(+) bacteria (Zhu et al., 2005; Ariki et al., 2008, respectively). Furthermore, the complement-mediated phagocytosis of bacteria by the hemocytes was also observed which indicates the presence of a primitive opsonic complement system in the horseshoe crab (Zhu et al., 2005). It seems that functional complement system had played a crucial role in innate immunity before the evolution of acquired immunity in vertebrates (Fujita et al., 2004).

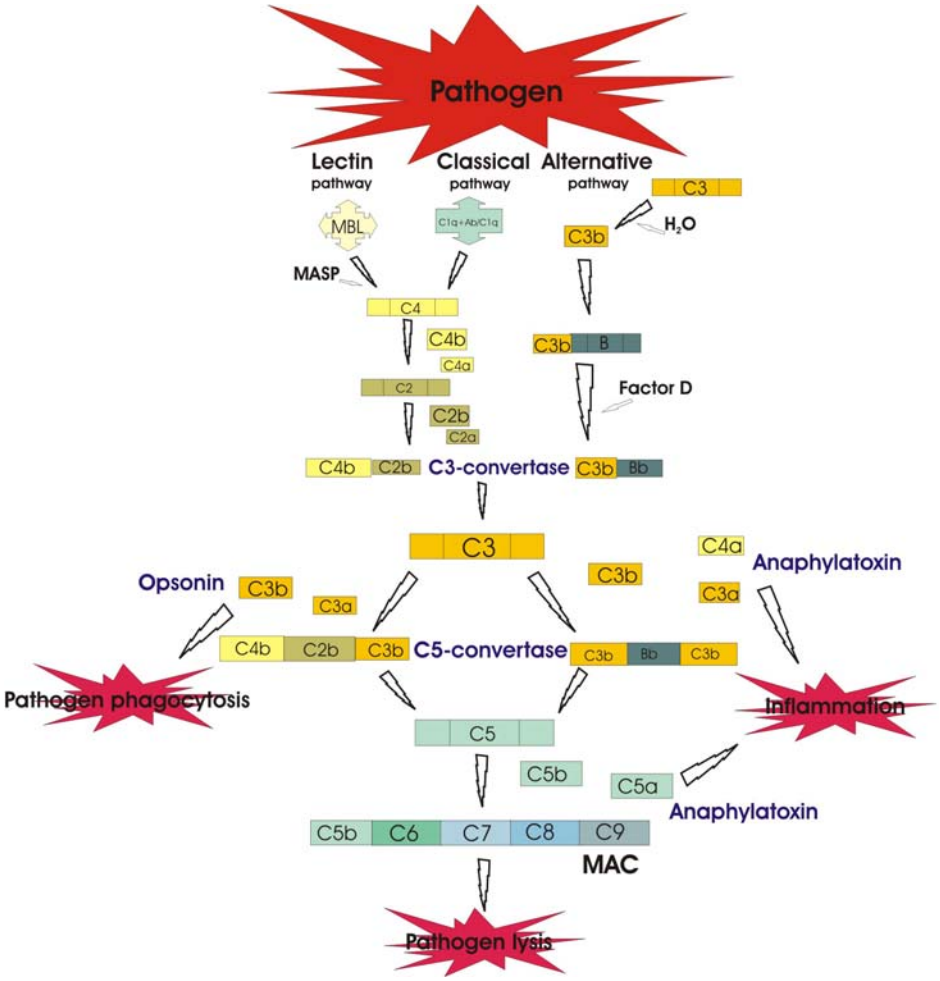


Figure 2: The activation of vertebrate complement by lectin, classical, and alternative pathway
The lectin pathway: the mannose-binding lectin (MBL) binds to the surface of pathogens. This recognition activates MASPs (MBL-associated serine proteases) that cleave C4 and newly generated C4b cleaves C2. Complex of C4bC2b is C3-convertase. The classical pathway: Antigen/antibody complex binds to C1q (part of C1 complex). C1q is also able to bind directly to pathogens surface. C1 complex is activated and it leads to identical cleavage of C4 and C2 as in the lectin pathway. In the alternative pathway, C3b or water reacted C3 bind to factor B, complex with factor B (C3bB) is formed. Part of factor B is cleaved by factor D and C3-convertase (C3bBb) is generated. If C3b does not bind to the pathogen, it is deactivated.

C3-convertase cleaves C3 into C3a (inflammatory peptide = anaphylatoxin) and C3b (a tag for phagocytosis). C3b also binds to C4bC2b or C3bBb and form C4bC2bC3b, C3bBbC3b complexes (C5-convertase), respectively. C5-convertase cleaves C5 into C5a (anaphylatoxin) and C5b which together with C6, 7, 8, and 9 is responsible for the formation of membrane-attack complex (MAC). MAC is effective primarily against Gram(-) bacteria and viruses.

2.2. Insect thioester containing proteins

Thioester containing proteins (TEPs) were first discovered in the genomes of the fruit fly *Drosophila melanogaster* (Lagueux et al., 2000) and the malaria mosquito *Anopheles gambiae* (Levashina et al., 2001; Moita et al., 2005). The best characterized molecule out of 15 TEPs of *A. gambiae* is aTEP1, a glycoprotein secreted by hemocytes into the hemolymph. Cleaved C-terminal part of aTEP1 binds to bacteria in a thioester-dependent manner (Levashina et al., 2001). Moreover, it binds directly to the surface of invading ookinetes of *Plasmodium berghei* and triggers their killing by lysis or melanization. Thus aTEP1 was proven as a determinant of the vectorial capacity in the malaria-transmitting mosquito (Blandin et al., 2004). Some other aTEPs were characterized as regulators of phagocytosis, where they play a specific role in defense against Gram(-) *Escherichia coli* (aTEP1 and aTEP3) and Gram(+) *Staphylococcus aureus* (aTEP4) (Moita et al., 2005).

In *D. melanogaster,* six dTEPs have been found, out of which four contain a conserved thioester motif (Lagueux et al., 2000; Stroschein-Stevenson, et al., 2006). The expression of dTEP1 and dTEP4 occurs mainly in hemocytes, dTEP1 is as well produced by fat body (Lagueux et al., 2000; Meister and Lagueux, 2003). Both dTEP2 and dTEP4 are markedly induced after a bacterial challenge in adults and larvae, whereas dTEP1 is up regulated only in larvae (Lagueux et al., 2000). dTEP6 possesses a modified sequence when compared with other *Drosophila* dTEPs because it lacks the thioester motif (Blandin and Levashina, 2004). This molecule, recently renamed as Mcr (macroglobulin complement related), was demonstrated to be specific for phagocytosis of the yeast *Candida albicans* by S2 *Drosophila* cells (Stroschein-Stevenson et al., 2006).

2.3. α_2-macroglobulins

Alpha$_2$-macroglobulins are broad-spectrum protease inhibitors with a well characterized function which is the clearance of active proteases from the tissue fluids. In the contrast to the active-site inhibitors that inactivate proteases via binding to their active

site, α_2Ms entrap the target protease leaving it active for the hydrolysis of small substrates. The protease entrapped within the α_2M molecule is subsequently removed from the circulation (Barrett and Starkey, 1973). The capture of protease and subsequent change of molecule conformation are possible due to α_2M unique domains. The target protease cleaves the α_2M in exposed and highly variable segment aptly termed as 'bait region'. The sequence variability of the 'bait region' enables α_2M to react with a wide variety of proteases (Sottrup-Jensen et al., 1989). The cleavage of α_2M in the'bait region' is followed by a rapid conformational change that leads to the exposure of the highly reactive thioester bond making it ready to create a new covalent bond fixing the entrapped protease (Figure 3) (Sottrup–Jensen, 1980; Armstrong and Quigley, 1999). Unreacted α_2M remains in plasma for a long time. On the other hand, the reacted α_2M-protease complex is promptly removed from the circulation via binding of carboxy terminal domain to the cell receptor (Sottrup-Jensen et al., 1996; Armstrong, 2006). The mammalian cell surface α_2M receptor has been identified as a low density lipoprotein receptor related protein (LRP-/α_2M-R) (Kristensen et al., 1990) and is present mainly in hepatocytes, macrophages and fibroblasts (Davidsen et al., 1985). In *Limulus*, the reacted α_2M is believed to be scavenged by hemocytes (Melchior et al, 1995). Small primary amines such as methylamine inactivate the reactive thioester bond and completely destroy its inhibitory activity (Sottrup-Jensen et al., 1980; Armstrong and Quigley, 1999).

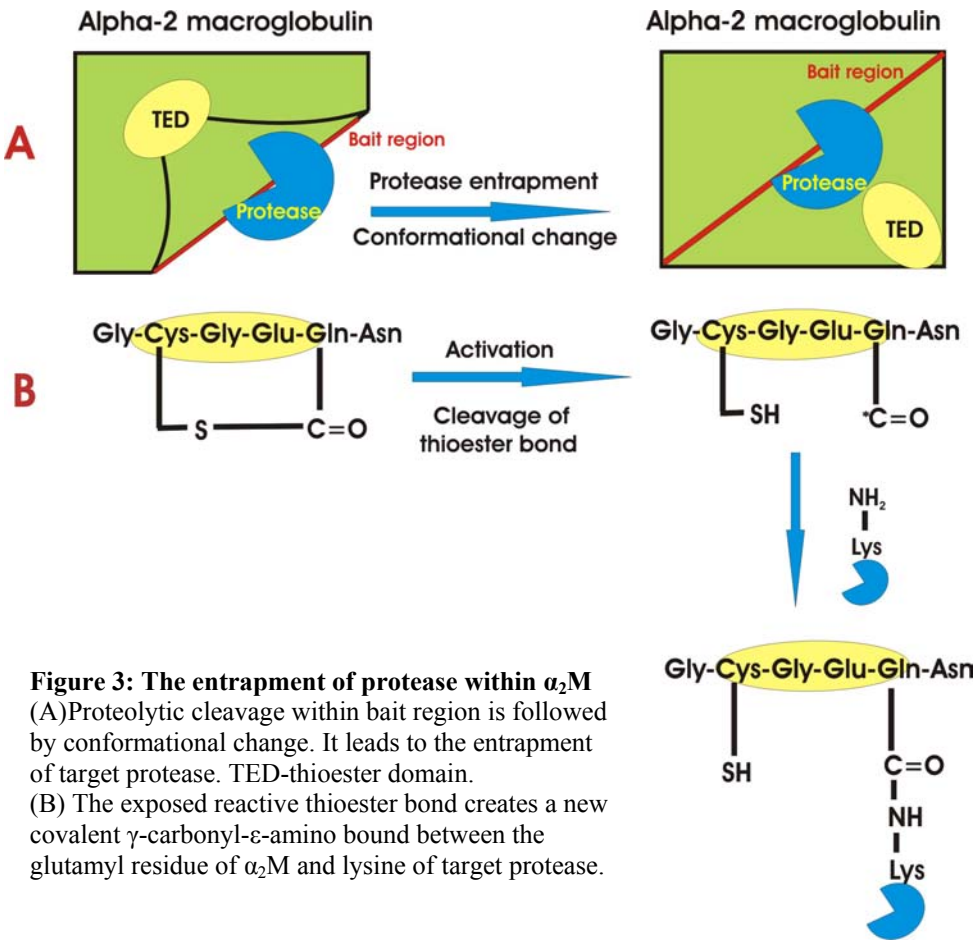


Figure 3: The entrapment of protease within α_2M
(A)Proteolytic cleavage within bait region is followed by conformational change. It leads to the entrapment of target protease. TED-thioester domain.
(B) The exposed reactive thioester bond creates a new covalent γ-carbonyl-ε-amino bound between the glutamyl residue of α_2M and lysine of target protease.

Alpha$_2$-macroglobulin is an abundant protein constituent of the plasma throughout many animals classes. It is found in nematodes, mollusks, arthropods, echinoderms, tunicates and vertebrates. Although α_2M is evolutionary conserved in the animal kingdom, the structural organization shows differences (Starkey and Barret, 1982). *Limulus* and other invertebrate α2Ms are mostly found as disulfide-linked homodimers (Armstrong et al., 1991; Husted et al., 2002), while a tetrameric structure is typical for the majority of vertebrates including human (Bowen et al., 1997). As an exception, monomeric α_2M was reported from the bullfrog *Rana catesebiana* (Rubenstein et al., 1993).

The main function of α_2M is to inhibit the wide range of proteases, including proteases from invading pathogens to prevent them to destruct surrounding tissues (Sottrup-Jensen et al., 1989). In humans, α_2Ms participate in the control of proteolytic activity, might serve as potent immunoregulators and have their roles in the biology of tumor cells (Chaudhuri, 1993). Although the role of α_2Ms in the innate immune defense is generally anticipated, only few examples describing the interaction of α_2M with

proteases of invading pathogens have been described. It was observed that α_2M binds onto *T. cruzii* protease found on the pathogen surface. Higher levels of α_2M were also observed in the mice infected with *T. cruzii* (Araújo-Jorge et al., 1992). *Pseudomonas aeruginosa* is another invasive pathogen producing protease, activity of which was found to be inhibited by α_2M of infected humans (Holder and Haidaris, 1979). In the shrimp *Marsupenaeus japonicus*, the expression of α_2M is significantly increased after the dosage of immune stimulant peptidoglycan which implies the importance of α_2M in the shrimp immunity (Rattanachai et al., 2004).

The genes coding for α_2M molecules were recently proved to be present in different bacteria species (Budd et al., 2004). The presented data strongly suggest that the α_2M genes have been horizontally acquired from the specific metazoan hosts. The function of α_2M in bacteria is not exactly known, but the authors assume their possible role in the distraction of host antimicrobial defense.

2.4. Structural aspects of the α_2-macroglobulin protein family

The three-dimensional structure of thioester proteins had not been solved for a long time due to their size and complexity. Recently, a progress in diffraction analysis and data processing made it possible to determine the 3D-structure of the native C3 and its major proteolytic fragment C3c, isolated from the human plasma (Janssen et al., 2005). The determination of human C3 structure allowed the detailed prediction of the location of corresponding structural domains in human α_2M (Doan and Gettins, 2007). Baxter et al. (2007) succeeded in the crystallization and diffraction of the full aTEP1r isoform from the malaria vector *A. gambiae* expressed in a baculovirus. The authors demonstrated that despite a limited sequence identity between aTEP1 and C3 (~25%) the structural relation of these two α_2M superfamily members is clear (Baxter et al., 2007; Blandin et al., 2008). The simplified view of C3 and TEP1 structural domains and the predicted structure of human α_2M (Doan and Gettins, 2007) are shown in Figure 4.

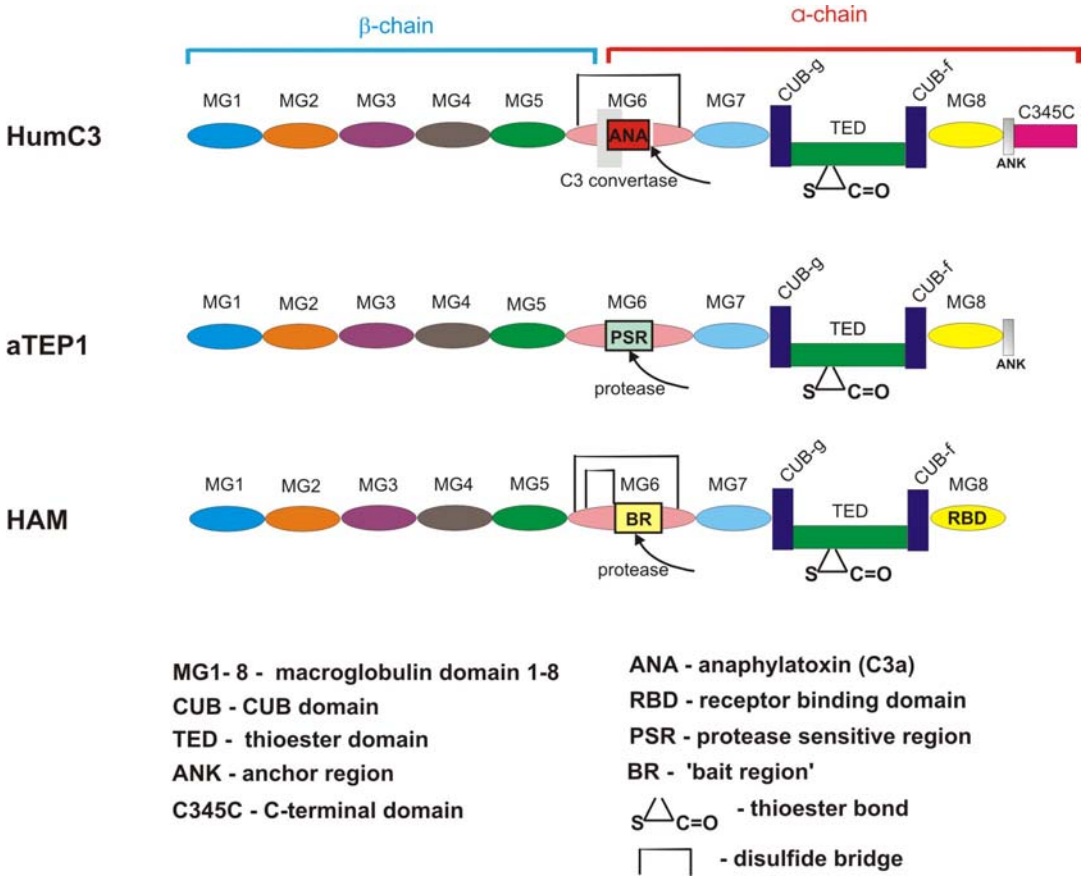


Figure 4: Structure of F-α_2M proteins
The large proteins of α2M family (1400-1800 residues) evolved from a core consisting of eight β-sheet macroglobulin domains (MG 1-8). They form two major rings named β and α that roughly correspond to the β-chain and α-chain of C3 post-translationally cleaved during secretion. The β-ring is composed from domains MG1-5 and a part of the MG6 which are attached to the α-ring forming the core of the protein. The α-ring is composed from other part of MG6, MG7, CUB domain with inserted α-helix thioester domain (TED) containing the reactive thioester bond. The C-terminal of α-ring in F-α_2M is formed by MG8 domain, which corresponds to the receptor binding domains in α_2Ms. The C3 molecule possesses an additional C-terminus domain named as C345C, which is a hallmark also for other complement component C4 and C5. The other functionally important region, namely the site of proteolytic activation of α_2M family proteins, occurs within the central MG6 domain. In the component C3 (C4, C5) this part is named as anaphylatoxin domain (C3a) which dissociates from the activated molecule upon action of protease complex named as C3 convertase. In TEPs, this region is termed as protease sensitive region as yet unknown protease cleavage is needed for aTEP1 activation. In protease inhibitors of α_2M class, this segment is highly variable and serves as a promiscuous 'bait' for a broad spectrum of proteases (adapted from Jannsen et al., 2005; Doan and Gettins, 2007; Baxter et al., 2007 and Blandin et al., 2008).

3. Ticks and their immune system

Ticks are blood-feeding ectoparasites and the most important disease vectors. They transmit wide variety of microorganisms, ranging from viruses, bacteria to protozoa, which are the causative agents of many animal and human diseases (Jongejan and Uilenberg, 2004). Transmitted pathogens have to be able to overcome several

barriers and to avoid the attack of tick immune system which, on the other hand, makes ticks resistant to infection of many other organisms. Compared to the other arthropods, little is known about the immune system of ticks and their immune interaction with pathogens (Sonenshine and Hynes, 2008).

Ticks, like other invertebrates, possess a humoral and a cellular innate immune response. Described molecules of tick humoral immunity involve AMPs, lysozymes, proteases, inhibitors of proteases and lectins.

The AMPs characterized in ticks were mainly of a defensin type. Tick defensins are produced in midgut, hemocytes, salivary glands and fat body (Nakajima et al., 2001; Nakajima et al., 2002; Zhou et al., 2007) and contribute to immune defense both in the midgut and hemolymph. Their production is up-regulated after a bacterial challenge (Johns et al., 2001a; Nakajima et al., 2003; Sonenshine et al., 2002). Defensins have been described in 11 ixodid and argasid tick species (Sonenshine and Hynes, 2008). The other antibacterial molecules of different type were characterized in *Boophilus microplus* and termed as microplusin and ixodidin (Fogaca et al., 2004; Fogaca et al., 2006, respectively).

Lysozyme that hydrolyses the cell wall peptidoglycans of Gram(+) bacteria was described to contribute to the midgut immunity in the soft tick *Ornithodoros moubata* (Kopáček et al., 1999; Grunclová et al., 2003). In the hard ticks, the lysozyme activity was detected in the hemolymph but not in the midgut (Simser et al., 2004). Large hemoglobin fragments were reported to contribute significantly to an antimicrobial defense in the tick gut (Fogaca et al., 1999; Sforca et al., 2005).

The lectin activity was detected in various tissues of several ticks (Grubhoffer et al., 2004) however, only few tick lectins have been sufficiently characterized. In our laboratory, we have described a lectin isolated from the plasma of *O. moubata* named Dorin M which is a fibrinogen-related protein (Kovář et al., 2000; Rego et al., 2006). Orthologs of this molecule (Ixoderin A and B) were identified to be expressed in hemocytes and salivary glands of the hard tick *I. ricinus* (Rego et al., 2005). The close relation of these molecules to tachylectins 5A, 5B from the horseshoe crab (Kawabata and Iwanaga, 1999) and mammalian ficolins (Matsushita and Fujita, 2001) together with our yet unpublished results suggest that fibrinogen-related lectins may play a role in a primitive complement-like system of ticks.

The protease inhibitors contribute to the inactivation of the pathogen proteases and have important functions in a variety of normal physiological pathways. In ticks, a

myriad of serin protease inhibitors has been described, having a role mainly as anticoagulant molecules in tick saliva (Maritz-Olivier et al., 2007). Nothing is known about the interaction of tick protease inhibitors with proteases of invading pathogens so far. We have isolated and characterized a protease inhibitor of α_2M class from *O. moubata* named TAM (Kopáček et al., 2000; Saravanan et al, 2003). A partial EST sequence of an α_2M molecule was identified also in the salivary glands transcriptome (sialome) of *I. scapularis* (Valenzuela et al., 2002).

Although some reports suggest existence of the PPO system or hemolymph clotting cascade in ticks (Soneshine and Hynes et al., 2008). No molecule related to the insect pro-phenoloxidase or horseshoe crab coagulogen has been identified yet.

The cellular part of tick immunity is represented by phagocytosis, nodulation and encapsulation. Hemocytes are the main participants in cellular response. Several types of hemocytes are recognized in ticks. Prohemocytes can be regarded as the stem cells, plasmatocytes and granulocytes serve for phagocytosis and encapsulation (Sonenshine, 1991). Unfortunately, little is known about the molecular mechanisms and factors involved in phagocytosis by tick hemocytes (Sonenshine and Hynes, 2008). A few of phagocytic studies have been done with hemocytes of the tick *O. moubata*, showing the phagocytosis of polystyrene beads (Inoue et al., 2001), the yeast *Candida haemulonii* (Loosová et al.; 2001) and the bacterium *Chryseobacterium indologenes* (Burešová et al., 2006) by tick hemocytes. Also several phagocytic *in vitro* studies were performed with tick cell lines, derived from embryonic cells (Kurtti et al., 1993; Kurtti and Keyhani, 2008). Some studies show that encapsulation and nodulation are also important in the immune defense of the ticks, only without melanization (Ceraul et al., 2002; Eggenberger et al., 1990).

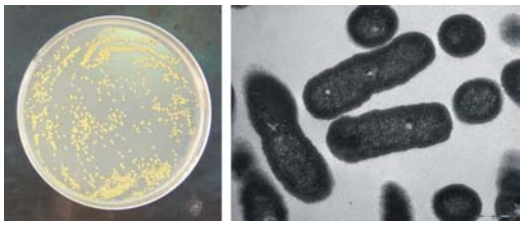
The most investigated process is an interaction between tick hemocytes and spirochetes *Borrelia* sp., the causative agents of the Lyme disease and relapsing fever. During their migration from the tick midgut to the salivary glands, the spirochetes are exposed to the cellular and humoral response. An uptake of spirochetes by the tick hemocytes was described (Coleman et al., 2001; Johns et al., 2001b). Kuhn et al. (1994) showed that *B. burgdorferi* is phagocytosed by both conventional and coiling phagocytosis. The formation of nodules was observed as well. The coiling phagocytosis is different from the conventional one; the pseudopod of hemocytes wrapped around spirochete in multiple layers form a pseudopod coil. This coil is translocated to the cytoplasm and subsequently degraded. Johns et al. (2001b) described that different tick

species exhibit a great difference in spirochete degradation. Also, tick response against different strains of *Borrelia* varied significantly (Dolan et al., 1998). It is evident that *Borrelia* is attacked by the immune system of tick. Unfortunately, the efficiency of immune system is not sufficient to prevent the successfull transmission of *Borrelia* spirochetes by ticks.

RESULTS AND DISCUSSION

1. ***Chryseobacterium indologenes*** **as a model tick pathogen (Paper I)**
2. **IrAM – an α_2-macroglobulin from *Ixodes ricinus* and its role in immunity (Paper II)**
3. **Unpublished data - The proteins of α_2-macroglobulin family in ticks *I. ricinus* and *I. scapularis***
4. **Supplemental material (Papers III, IV)**

PAPER I



Veronika Burešová, Zdeněk Franta, Petr Kopáček, 2006

A comparison of *Chrysobacterium indologenes* pathogenicity to the soft tick *Ornithodoros moubata* and hard tick *Ixodes ricinus*

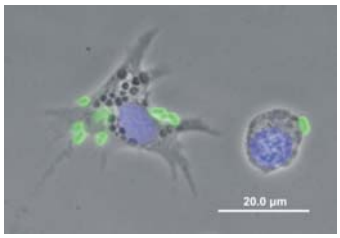
J Invertebr Pathol 93: 96-104

Abstract

A yellow-pigmented Gram-negative bacterium, *Chryseobacterium indologenes*, was found in the gut contents of about 65% of soft ticks *Ornithodoros moubata* from a perishing laboratory colony. The isolated putative pathogen, *C. indologenes*, was susceptible to cotrimoxazol and addition of this antibiotic (Biseptol 480) to the blood meal significantly decreased the tick mortality rate. The artificial infection of healthy *O. moubata* by membrane feeding on blood contaminated with *C. indologenes* was lethal to all ticks at concentrations $\geqslant 10^6$ bacteria/ml. On the contrary, a similar infection dose applied to the hard tick *Ixodes ricinus* by capillary feeding did not cause significant mortality. Examination of guts dissected from infected *O. moubata* and *I. ricinus* revealed that *C. indologenes* was exponentially multiplied in the soft tick but were completely cleared from the gut of the hard ticks within 1 day. In both tick species, *C. indologenes* were found to penetrate from the gut into the hemocoel. The phagocytic activity of hemocytes from both tick species was tested by intrahaemocoelic microinjection of *C. indologenes* and evaluated by indirect fluorescent microscopy using antibodies raised against whole bacteria. Hemocytes from both tick species displayed significant phagocytic activity against *C. indologenes*. All *O. moubata* injected with *C. indologenes* died within 3 days, whereas the increase of the mortality rate of *I. ricinus* was insignificant. Our results indicate that hard ticks possess much more efficient defense system against infection with *C. indologenes* than the soft ticks. Thus, *C. indologenes* infection has the potential to be a relevant comparative model for the study of tick immune reactions to transmitted pathogens.

Keywords: Tick; Pathogen; *Chryseobacterium indologenes*; *Ornithodoros moubata*; *Ixodes ricinus*; Blood-feeding; Innate immunity

PAPER II



Veronika Burešová, Ondřej Hajdušek, Zdeněk Franta, Daniel Sojka, Petr Kopáček, 2009

IrAM - An α_2-macroglobulin from the hard tick *Ixodes ricinus*: characterization and function in phagocytosis of potential pathogen *Chryseobacterium indologenes*

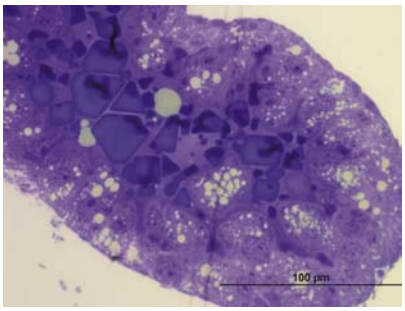
Dev Comp Immunol 33: 489-498

A B S T R A C T

The universal protease inhibitors of the α_2-macroglobulin (α_2M) family are evolutionarily conserved constituents of innate immunity, presumably because they guard organisms against undesired proteolytic attacks of a different origin. Here, we determined the primary structure of α_2-macroglobulin from the hard tick *Ixodes ricinus* (IrAM) by sequencing of overlapping PCR products. Predicted disulfide and glycosylation patterns, post-translational cleavage and alternative splicing within its 'bait region' demonstrate that IrAM is closely related to the α_2-macroglobulin from the soft tick *Ornithodoros moubata*. The IrAM message is expressed in all tick developmental stages and tissues, except for the gut, and the protein was detected to be mainly present in the hemolymph. Silencing of IrAM by dsRNA interference markedly reduced the phagocytosis of a potential pathogen, *Chryseobacterium indologenes*, by tick hemocytes both *in vitro* and *in vivo*. In contrast, phagocytosis of the Lyme disease spirochete *Borrelia burgdorferi* or a commensal bacteria *Staphylococcus xylosus* was not affected by the IrAM knock-down. Similar results were obtained upon deactivation of all thioester proteins in tick hemolymph by methylamine. We have further demonstrated that phagocytosis of *C. indologenes* is dependent on an active metalloprotease secreted by the bacteria. These data indicate that interaction of tick α_2-macroglobulin with a protease of an invading pathogen is linked with cellular immune response.

SUPPLEMENTAL MATERIAL

PAPER III



Daniel Sojka, Ondřej Hajdušek, Jan Dvořák, Mohammed Sajid, Zdeněk Franta, Eric L. Schneider, Charles S. Craik, Marie Vancová, Veronika Burešová, Matthew Bogyo, Kelly B. Sexton, James H. McKerrow, Conor R. Caffrey, Petr Kopáček, 2007

IrAE: an asparaginyl endopeptidase (legumain) in the gut of the hard tick *Ixodes ricinus*

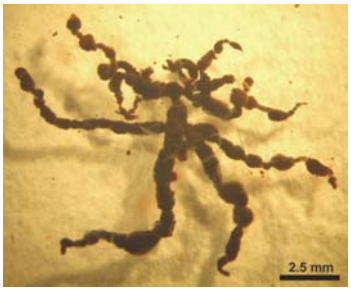
Int J Parasitol 93: 713-724

Abstract

Ticks are ectoparasitic blood-feeders and important vectors for pathogens including arboviruses, rickettsiae, spirochetes and protozoa. As obligate blood-feeders, one possible strategy to retard disease transmission is disruption of the parasite's ability to digest host proteins. However, the constituent peptidases in the parasite gut and their potential interplay in the digestion of the blood meal are poorly understood. We have characterised a novel asparaginyl endopeptidase (legumain) from the hard tick *Ixodes ricinus* (termed IrAE), which we believe is the first such characterisation of a clan CD family C13 cysteine peptidase (protease) in arthropods. By RT-PCR of different tissues, IrAE mRNA was only expressed in the tick gut. Indirect immunofluorescence and EM localised IrAE in the digestive vesicles of gut cells and within the peritrophic matrix. IrAE was functionally expressed in *Pichia pastoris* and reacted with a specific peptidyl fluorogenic substrate, and acyloxymethyl ketone and aza-asparagine Michael acceptor inhibitors. IrAE activity was unstable at pH $\geqslant$ 6.0 and was shown to have a strict specificity for asparagine at P1 using a positional scanning synthetic combinatorial library. The enzyme hydrolyzed protein substrates with a pH optimum of 4.5, consistent with the pH of gut cell digestive vesicles. Thus, IrAE cleaved the major protein of the blood meal, hemoglobin, to a predominant peptide of 4 kDa. Also, IrAE *trans*-processed and activated the zymogen form of *Schistosoma mansoni* cathepsin B1 – an enzyme contributing to hemoglobin digestion in the gut of that bloodfluke. The possible functions of IrAE in the gut digestive processes of *I. ricinus* are compared with those suggested for other hematophagous parasites.

Keywords: Asparaginyl endopeptidase; Legumain; Protease; Tick; Midgut; Hemoglobin digestion; *Ixodes ricinus*

PAPER IV



Ondřej Hajdušek, Daniel Sojka, Petr Kopáček, Veronika Burešová, Zdeněk Franta, Ivo Šauman, Joy Winzerling, Libor Grubhoffer, 2009

Knockdown of proteins involved in iron metabolism limits tick reproduction and development

Proc Natl Acad Sci 106: 1033-1038

Ticks are among the most important vectors of a wide range of human and animal diseases. During blood feeding, ticks are exposed to an enormous amount of free iron that must be appropriately used and detoxified. However, the mechanism of iron metabolism in ticks is poorly understood. Here, we show that ticks possess a complex system that efficiently utilizes, stores and transports non-heme iron within the tick body. We have characterized a new secreted ferritin (FER2) and an iron regulatory protein (IRP1) from the sheep tick, *Ixodes ricinus*, and have demonstrated their relationship to a previously described tick intracellular ferritin (FER1). By using RNA interference-mediated gene silencing in the tick, we show that synthesis of FER1, but not of FER2, is subject to IRP1-mediated translational control. Further, we find that depletion of FER2 from the tick plasma leads to a loss of FER1 expression in the salivary glands and ovaries that normally follows blood ingestion. We therefore suggest that secreted FER2 functions as the primary transporter of non-heme iron between the tick gut and the peripheral tissues. Silencing of the *fer1*, *fer2*, and *irp1* genes by RNAi has an adverse impact on hatching rate and decreases postbloodmeal weight in tick females. Importantly, knockdown of *fer2* dramatically impairs the ability of ticks to feed, thus making FER2 a promising candidate for development of an efficient anti-tick vaccine.