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Differential expression of tick *Ixodes ricinus* genes induced by blood feeding or infection: genetic analysis of ML domain containing proteins

Ph.D. Thesis

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Annotation

ML (MD-2-related lipid-recognition) domain containing proteins are recognized as immune-related molecules. They do not belong among well-studied proteins in ticks although their occurence is quite often. Generally, ML proteins are involved in innate immunity processes, lipid binding and transport. Usually, expression of tick ML domain containing proteins is induced by blood feeding. Two members of the ML protein family, ML-domain containing protein and Der-p2 allergen-like protein were isolated from *Ixodes ricinus* and characterized for the first time.

Declaration [in Czech]

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. Jana Plchová

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. **Horáčková, J.**, Rudenko, N., Golovchenko, M., Grubhoffer, L., 2010. Der-p2 (*Dermatophagoides pteronyssinus)* allergen-like protein from the hard tick *Ixodes ricinus* – a novel member of ML (MD-2-related lipid-recognition) domain protein family. Parasitology 137, 1139-1149 (IF = 2.522).

Jana Plchová (Horáčková) participated in design of experimental procedure, tick collection, nucleic acids extraction and handling, gene isolation, bioinformatics/sequence analysis, expression and purification of the recombinant protein, antimicrobial and IgE binding activity determination, preparation of results for publication, manuscript writing and its revision.

II. **Horáčková, J.**, Rudenko, N., Golovchenko, M., Havlíková, S., Grubhoffer, L., 2010. *IrML* – a gene encoding a new member of the ML protein family from the hard tick, *Ixodes ricinus*. Journal of Vector Ecology 35, 410-418 (IF = 1.256).

Jana Plchová (Horáčková) participated in design of experimental procedure, tick collection, nucleic acids extraction and handling, gene isolation, bioinformatics/sequence analysis, preparation of in situ hybridization probes and in situ hybridization technique performing, preparation of results for publication, manuscript writing and its revision.

Contents

1. Introduction and literature review

1.1 Introduction

Ticks form the most important group of arthropods that transfers the greater variety of pathogens than any other arthropod vector group. They are ectoparasitic organisms that are dependent on blood intake. During the blood feeding, many changes occur in the tick's body on morphological, physiological and molecular level. The potential role of differentially expressed genes in the physiology of ticks underlies the host response of the tick to blood meal or infection. Many of the genes that were induced in castor bean tick *Ixodes ricinus* by blood meal or pathogen invasion fit into the current conceptual framework of host defense. The elucidation of inducible genes will provide a better understanding of the competence of *I. ricinus* as a vector of different pathogens.

In castor bean tick, differentially expressed genes were observed in blood-fed and *Borrelia burgdorferi* infected blood-fed ticks (Rudenko et al. 2005). Two cDNA libraries representing transcripts of expressed genes were prepared by subtractive hybridization in our laboratory. The first library contained gene transcripts of blood-fed ticks and the second library was comprised of *B. burgdorferi* infection-associated transcripts. The analysis of detected differentially expressed genes resulted in their assignment into several groups: genes involved in pathogen recognition and defense, oxidative stress response, digestion and pathogen transmission. The group of genes related to pathogen recognition and defense includes also the genes encoding proteins that belong to the ML domain protein family. These proteins with ML domain are often involved in lipid recognition and/or transport, especially in recognition of pathogen related molecules (Inohara and Nuñez 2002). Since the occurrence of proteins with ML domain is quite often, it is possible that they might be involved in different mechanisms of immune response of wide range of organisms and definitely deserve intensive study that might elucidate their function in tick's organism.

1.2 Ticks

1.2.1 General information

Ticks are obligate hematophagous arthropods occurring in almost every region of the world. They inhabit countries with tropical, subtropical, mild, and arctic climate. Most ticks parasitize warm-blooded organisms (mammals, birds) but some also attack several cold-blooded animals (reptiles, amphibians). Due to their occurrence, ticks are important vectors of pathogens causing a wide range of diseases worldwide. Ticks are able to transmit different viruses, bacteria, rickettsiae, protozoans and fungi representing a higher risk for more organisms than the majority of studied insect vectors. Taxonomically, almost 900 tick species belong to the subclass Acari and are divided into three families: Ixodidae ("hard ticks", 713 species of 13 genera), Argasidae ("soft ticks", 185 species of 4 genera) and Nuttalliellidae (1 species) (Table 1). Mostly the representatives of family Ixodidae occur in Europe.

1.2.2 Overview of tick anatomy and physiology

Ticks' body is divided into capitulum and idiosoma. The idiosoma is distinguished to podosoma with chelicerae, palps, hypostome and 4 pairs of legs, and opisthosoma with anal aperture. The hypostome is modified specifically for blood-feeding and is essential for the attachment to the host. Chemosensillas, mechanosensillas and eyes or photoreceptors are located on the capitulum. Ticks also have a special sensory organ - Haller's organ – on the tarsus of the leg I. This organ provides information about host location, host odors, or detection of pheromones. Cuticle, secreted by epidermis, covering whole tick body, serves as an exoskeleton (like in other arthropods); a layer of cuticle called procuticle, specifically its outer part, becomes sclerotized in certain parts and forms sclerites. The biggest sclerite, scutum, covers the anterior part of the body and protects the dorsal side of it. The major components of the cuticle are proteins and chitin; lipids represent a minor part.

For pathogen transmission, the alimentary system (especially midgut), hemolymph and salivary glands are the most important inner organs. Pathogens get into the tick's body via foregut. They enter hemocoel or hemolymph that washes every inner organ, from the midgut and continue to their target organ.

Table 1. Overview of tick genera (Barker and Murrell 2004).

Family	Subfamily (subgroup)	Genera
Ixodidae ("hard ticks")	Ixodinae (Prostriata)	*Ixodes*
	Amblyomminae (Metastriata)	*Amblyomma*
	Haemaphysalinae (Metastriata)	*Haemaphysalis*
	Hyalomminae (Metastriata)	*Hyalomma*
	Bothriocrotoninae (Metastriata)	*Bothriocroton*
	Rhipicephalinae (Metastriata)	*Rhipicephalus*
		Rhipicentor
		Dermacentor
		Cosmiomma
		Cornupalpatum
		Margaropus
		Nosomma
		Anomalohimalaya
Argasidae ("soft ticks")	Argasinae	*Argas*
	Ornithodorinae	*Ornithodoros*
		Otobius
		Carios
Nuttalliellidae	Nuttaliellinae	*Nuttalliella*

The alimentary system is divided into three parts: foregut, midgut and hindgut. The midgut is the biggest part of the alimentary system where digestion takes place. The midgut's branches serve as storage place. The midgut is formed by epithelial and muscle cells. Contrary to insect, the digestion in ticks is an intracellular process (with the exception of the intraluminal digestion of erythrocytes). Several types of cells can be distinguished in its epithelial layer, i.e., undifferentiated, digestive and secretory. The uptaken blood gets into the digestive cells by pinocytosis or endocytosis. Feeding is divided into several phases. The first, preparatory phase starts after attachment to the host, usually in 12-36 hours. No ingestion comes on and tick prepares feeding lesion first of all. Then, the growth phase (or slow feeding) takes place during few days and ingested blood is digested. The undifferentiated cells of the midgut epithelium grow and give rise to digestive and secretory cells. Creation of a peritrophic

membrane, that protects the midgut epithelium during injury and pathogens penetration, was detected in some species. After slow feeding period, rapid feeding period or repletion in mated females starts. The female increases its weight approximately 100 times in comparison to unfed state. Ingested blood is stored in midgut cells for further processing. Probably, vitellogenin producing cells grow up during this phase. Afterward, the tick drops off its host and females prepare for oviposition; in cases of larva and nymph molting occurs. The lysis of intracellular stored hemoglobin is much faster at this stage.

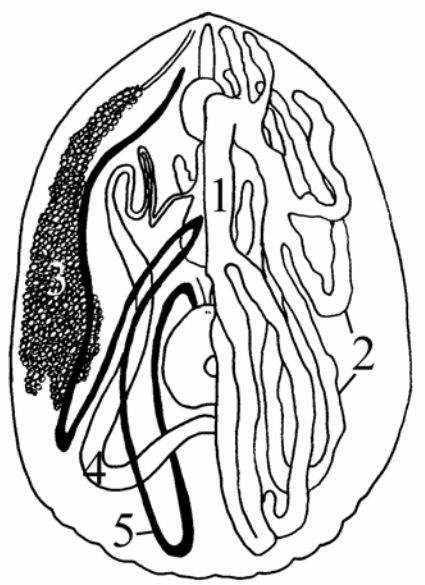

Fig. 1. Scheme of hard tick's female internal organs. 1 – midgut, 2 – midgut diverticulum, 3 – salivary gland, 4 – ovary, 5 – Malphigian tubules. Adapted from Sonenshine 1991.

Hemolymph surrounds all inner organs and consists of plasma and hemocytes. Plasma, besides water, contains proteins, lipids, amino acids, carbohydrates, inorganic salts or certain pigments. Hemocytes are distinguished into several types: prohemocytes, plasmatocytes, granulocytes and spherulocytes. They are described in the chapter dedicated to tick immunity.

The biggest tick's gland is a pair of salivary glands consisting of a large cluster of acini joined together by a system of ducts. Several types of acini are distinguished. Type I acini (or agranular acini) mouth through short acinar ducts directly into the main salivary duct. These agranular acini have highly vacuolated central cells in the middle. They are responsible for the uptake of atmospheric moisture by unfed ticks and so for maintenance of water balance. Other acini types are granular due to the content of large granules. In soft ticks, only a granular acinus type occurs (type II acinus). As many as three different types of acini, types II, III and in males also type IV, are described in hard ticks. They contain granules with different compounds and occur in clusters that are usually opened into secondary ducts joined with the main salivary duct. Several

cells of these acini are able to eliminate excessive water or secrete cement (in some species) in the first phase of feeding. During this process salivary glands are also enlarged several times (Sonenshine 1991).

1.2.3 Tick immunity

As was mentioned, ticks transmit great amount of various pathogens. All parasitic organisms get into the tick body usually in two main ways: through injury and during blood intake. To survive, tick has to defend itself against a high dosage of parasites. Ticks, as other invertebrates, did not develop adaptive immunity but use only innate immune responses. The first obstacle for parasites is the physical barrier of the tick's body – epithelia beneath cuticle, in tracheae and in digestive tract. Many of immune compounds are accumulated or produced in the tick's midgut through which the majority of pathogens enter the tick's body at the beginning of tick colonization.

Briefly, as with other arthropods, ticks are shown to possess two sets of immune responses. The first are cellular responses in ticks which include encapsulation, nodulation and phagocytosis. Humoral responses are the second set of immune responses in ticks and comprise the proteins that represent the molecular factors of self/non-self recognition (innate immunity) either involved in defense reactions or pathogen pattern recognition, as well as the effector molecules with enzymatic activities (proteases in the cascade of inactive enzymes, protein inhibitors of proteases). Understanding of tick-immunity is important determination of the host-pathogen interactions that facilitate or limit disease transmission.

Tick innate immune system must be able to distinguish between self and non-self molecules. This recognition is carried out by conserved pattern recognition receptors (PRRs) whose ligands are microbial components with pathogen-associated molecular patterns (PAMPs). PAMPs represent non-self, usually lipids and carbohydrate molecules with typical occurrence in cell surfaces of bacteria and fungi. They often act as opsonins and so trigger cellular immune processes. For example, several lectins or peptidoglycan recognition proteins belong to PRRs (McGuinness et al. 2003; Dziarsky and Gupta 2006). As described in *Drosophila*, binding of PAMPs to PRRs leads to activation of Toll or Imd signaling pathway and further to activation of transcription of NF-

κB controlled immune response genes, often genes encoding components of humoral immunity (Michel et al. 2001; Tanji and Ip 2005).

The major participants of cellular immune response are hemocytes and they deserve a closer look. Since hemolymph is surrounding all internal organs, hemocytes act in any compartment of the tick's body. Four types of hemocytes were described in ticks: prohemocytes, plasmatocytes, granulocytes (type I and II) and spherulocytes (Sonenshine and Hynes 2008). Rounded prohemocytes (6-8 μm) are the smallest hemocytes with high nuclear/cytoplasmic ration, and without granules. They are considered to be stem cells from which other hemocytes differentiate. Plasmatocytes (9-20 μm) often possess the extensions of plasma membrane and undertake the function of phagosomes. Larger granulocytes (15-20 μm) are also phagocytic cells. They also can have filopodia. Type I granulocytes contain electron-dense granules and granules with inner tubular structure while only electron-dense granules occur in type II granulocytes. The last type, spherulocytes (8-10 μm) have numerous large membrane bounded granules with tubular structure (Borovičková and Hypša 2005).

As it was mentioned above, plasmatocytes and granulocytes I are able to phagocytize and encapsulate. Phagocytosis is a process that is comprised of several steps: recognition of microorganism, binding to the surface receptor, signal transduction and engulfment of foreign particle (Stuart and Ezekowitz 2005). Contrary to insect, no information is available about the process of phagocytosis in ticks. It was observed that tick hemocytes phagocytize mainly Gram positive bacteria, spirochetes, and also fungi (Sonenshine and Hynes 2008). In soft tick *Ornithodoros moubata* the ability of hemocytes to phagocytize bacterium *Chryseobacterium indologenes* (Burešová et al. 2006) and yeast *Candida haemulonii* (Loosová et al. 2001) was observed. Hemocytes of hard tick *I. ricinus* also engulf *C. indologenes* (Burešová et al. 2006). Interaction of hemocytes with Lyme disease spirochetes *Borrelia burgdorferi* that led to up to 6-fold increase of number of phagocytic cells in *I. scapularis* was described (Coleman 1997; Johns et al. 2000, 2001). On the contrary, spirochetes in non-vector tick species *Dermacentor variabilis* were cleared by mechanism other than phagocytosis. Two ways of *B. burgdorferi* phagocytosis by tick hemocytes were described in *I. scapularis* and *I. ricinus*: conventional

and coiling phagocytosis (Rittig et al. 2006; Grubhoffer et al. 2005). Production of reactive oxygen species during phagocytosis of bacteria by *R. microplus* hemocytes was also reported (Pereira et al. 2001).

Nodulation is another defense mechanism mainly used against large numbers of bacteria. During nodule formation microorganisms are captured in a coagulum of granular cells coated by hemocytes (Taylor 2006). Larger pathogens like nematodes are destructed by encapsulation, mechanism in which foreign particles are surrounded with a multilayer capsule composed of hemocytes (Eggenberger et al. 1990). Nodulation and encapsulation have been observed in *D. variabilis* so far (Eggenberger et al. 1990; Ceraul et al. 2002).

The second part of innate immunity, humoral immunity, is formed by three main mechanisms: humoral encapsulation, hemagglutination, and production of antimicrobial peptides (AMPs). Humoral encapsulation proceeds in a similar way as encapsulation by hemocytes, but is evoked by prophenoloxidase system. Prophenoloxidase system is well-studied in insect but not in ticks. To date, phenol oxidase activity was detected only in hemolymph of *O. moubata* nymphs (Kadota et al. 2002). Hemagglutination is mediated by protein or glycoprotein molecules that bind sugars. Such molecules are known as lectins or agglutinins. The first described tick lectins were found (but not isolated) in hemolymph of *I. ricinus*, *O. tartakovskyi*, *O. papillipes* and *Argas polonicus* (Grubhoffer et al. 1991). Hemagglutination activity was also revealed in hemolymph and extracts of gut and salivary glands of the hard tick *Rhipicephalus appendiculatus* (Kamwendo et al. 1993). In *I. ricinus*, hemagglutination activity was observed in midgut homogenate (Uhlíř et al. 1996). First identified tick lectin molecule was glycoprotein Dorin M from hemolymph of *O. moubata*. The primarily site of Dorin M expression was defined to be hemocytes but it was also detected in tick salivary glands. Most probably Dorin M is involved in recognition of non-self molecules (Kovář et al. 2000; Rego et al. 2006). Lectins with fibrinogen-related domain were identified in *O. moubata* and *I. ricinus* (Rego et al. 2005). Another type of lectin with possible function in innate immunity, galectin, was found in various organs of *O. moubata* (Huang et al. 2007).

Members of α2-macroglobulin superfamily with relationship to complement system in vertebrates (α_2-macroglobulins, C3 complement

components) and insect (thioester-containing and macroglobulin-related proteins) were also described in ticks (Burešová et al. 2011). For example, the alpha2-macroglobulin of *I. ricinus* is involved in phagocytic process of bacterium *C. indologenes* by tick hemocytes (Burešová et al. 2009).

The most distinctive part of humoral immunity is represented by antimicrobial peptides (AMPs). They consist rather diverse group of small peptides (usually 4-20 kDa) with significant difference in primary and secondary structure that enables them to interact with a wide variety of microorganisms. Antimicrobial peptides interact with invaders through their plasma membrane or cell wall. Although many AMPs have been studied, their mode of action and exact functions still remain unknown (Taylor 2006). The most studied types of AMPs are defensins, lysozymes, protease inhibitors and some others.

Defensins represent the most widespread and the most studied group of AMPs in ticks. These cysteine-rich mostly cationic peptides are synthesized as prepropeptides and processed to the small mature peptides (3-6 kDa). Six conserved cysteine residues are part of the defensins' primary structure. They are vital for the formation of disulfide bridges and stabilizing of their specific fold. Expression of defensins is usually up-regulated by bacterial challenge, especially by Gram-positive bacteria (e.g. *Micrococcus luteus*, *Staphylococcus aureus* in *O. moubata; S. aureus, Bacillus subtilis, Corynebacterium renale* in *I. persulcatus*) but in lower level also by Gram-negative bacteria (e.g. *E.coli* in *I. persulcatus* or *Haemaphysalis longicornis*) (Nakajima et al. 2002a; 2003; Lai et al. 2004; Zhou et al. 2007). Induction of defensin gene expression by blood feeding was described in soft and in hard ticks (Nakajima et al. 2001, 2002b; Rudenko et al. 2005). Site of defensins production (mainly in hemolymph and midgut) also indicates the site of action. Until date, defensins were identified in 6 soft and 11 hard tick species, usually in more than one isoform (Rudenko et al. 2007; Sonenshine and Hynes 2008; Saito et al. 2009; Chrudimská et al. 2010).

Several small antimicrobial peptides without similarity either to defensins or to other AMPs were discovered in different tick species. They show antimicrobial activity mostly against Gram-positive bacteria. Some of them were also active against Gram-negative bacteria and fungi. Cysteine-rich

microplusin from *R. microplus* (Fogaça et al. 2004), histidine-rich hebraein from *Amblyomma hebraeum* (Lai et al. 2004), ixodidin from *R. microplus* (Fogaça et al. 2006), ixosins from *I. sinensis* (Yu et al. 2006; Liu et al. 2008) or longicornsin from *H. longicornis* (Lu et al. 2010) belong to this group of AMPs.

Lysozymes hydrolyze the β-1,4-glycosidic bonds between N-acetylmuramic acid and N-acetyl-D-glucosamine in cell wall peptidoglycans of Gram positive bacteria. Lysis activity of lysozymes from *Alveonasus lahorensis*, *O. moubata*, *O. papillipes*, *Hyalomma asiaticum* and *I. persulcatus* against Gram positive bacterium was described (Podboronov 1991). The level of lysozymes in hemolymph usually increases after the bacterial infection, as was observed in *D. variabilis* and *O. moubata* (Simser et al. 2004). In soft tick *O. moubata* the higher level of the AMP was also detected in midgut under the same conditions (Grunclová et al. 2003).

Many types of protease inhibitors have been identified and characterized in different tick species. A big group of inhibitors is connected with suppression of host defensive processes, prolongation of blood coagulation time as well as with blood digestion (e.g. serine protease inhibitors, trombin inhibitors). However, several inhibitors play significant role in tick immune reactions (cystatins – cysteine protease inhibitors) (Sonenshine and Hynes 2008).

1.2.4 Tick life cycle

During ontogenesis, ticks go through several developmental stages: egg, larva, nymph (several nymphal stages occur in argasid ticks) and adult. Every motile stage seeks for a host to feed on it. It needs energy and nutrients from blood for molting to the following stage and in case of adult females for oviposition or, in males (with exception of tick from *Ixodes* genus), for gametogenesis. Contrary to insects, ticks' life cycle lasts several years. Significant variations in their duration were observed between ixodids and argasids. Hard ticks usually feed on hosts for several days whereas soft ticks only several tens of minutes. Another difference is in the number of egg batches – ixodids lay eggs only once and dies whereas argasids do this several times during a life time (unique batches have smaller number of eggs). The majority of ixodid ticks attacks different hosts in different developmental stage, and thus

they have 3-host life cycle. There also exist certain exceptions – several species feed only on two or even one host. The life cycle of soft ticks is multi-host because of the existence of several nymphal instars and the repeating feedings of adults (Sonenshine et al. 2002).

1.2.5 *Ixodes ricinus*

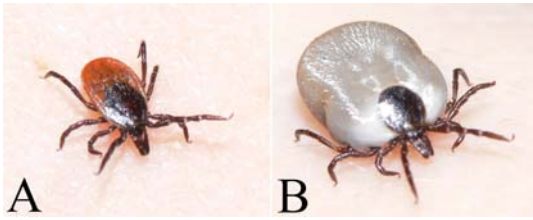

Fig. 2. Unfed (A) and engorged (B) female of European sheep or castor bean tick *I. ricinus.*

Castor bean tick, *Ixodes ricinus*, also known as a sheep tick, is spread across whole Europe, from Iceland to Russia. The exception is Mediterranean region, where living conditions for the ticks are unfavorable due to low humidity. Ticks distribution reaches the northern Africa in the south and Asia (Iran) in the east. They occur from lowlands to mountains. Few decades ago, ticks habitat was limited to altitude of 700-800 m.a.s.l. Nowadays ticks can be found even at altitude of 1250 m.a.s.l. (Materna et al. 2007). *I. ricinus* inhabits forests, woodlands and meadows or heaths. It has a typical 3-host life cycle. After hatching from eggs in leaf litter, larvae spread into the vegetation and parasite on the first host. Engorged, they drop off the host and molt to nymphs. Nymphs seek the second host, feed and molt again. Adults search for the third host, mate and females feed (males do not need to feed whereas their gametogenesis begins with the molting, not after feeding as in other genera); after dropping off females find optimal environment, lay eggs and die (Sonenshine 1991). The life cycle lasts under favorable conditions 1 year but in Central Europe it takes usually 3 years because of a winter diapause in each developmental stage. Over 300 animal species can be parasitized by *I. ricinus.* Typical hosts are small mammals, birds and lizards for larvae and nymphs; bigger animals like deer, sheep, cattle and other ruminants belong to typical hosts for adult ticks. Humans are rather occasional hosts.

I. ricinus is a vector of several pathogens (viruses, bacteria, rickettsiae, protozoans) that can cause a variety of diseases. In humans the most serious are Lyme disease and tick-borne encephalitis.

Lyme disease, a systemic illness, is caused by spirochetes from *Borrelia burgdorferi* sensu lato complex. Development of this disease usually involves three stages: early localized, early disseminated and late Lyme disease; patients usually have cutaneous, neurologic, musculoskeletal, cardiac and ophthalmologic manifestations (Sigal 2001; Brouqui et al. 2004). The causative agent infects the tick during its feeding on an infected host and colonizes tick gut. The receptor for one of borrelial surface antigen (exactly Outer Surface Protein A), TROSPA (Tick Receptor for OspA), was described in north American tick *I. scapularis*. Most probably it helps spirochetes to persist in the tick's body (Pal et al. 2004). During tick feeding, spirochetes migrate from the gut through hemolymph into salivary glands and further, infecting host. Considering the human sensitivity to *B. burgdorferi* s.l. spirochetes the complex of 18 *Borrelia* genospecies recognized till date can be divided into two major groups. The first group consists of 9 species that have not yet been reported in or isolated from humans. This group includes *B. americana, B. andersonii, B. californiensis, B. carolinensis, B. japonica, B. tanukii, B. turdi, B. sinica,* and *B. yangtze*; and the second group is formed by 9 species with pathogenic potential. This group includes *B. afzelii, B. bavariensis, B. bissettii, B. burgdorferi* s.s., *B. garinii, B. kurtenbachii, B. lusitaniae, B. spielmanii* and *B. valaisiana* (Rudenko et al. 2011).

Tick-borne encephalitis virus (TBEV) belongs to genus *Flavivirus*, family *Flaviviridae*. TBEV is a causative agent of a serious neuroinfection in Europe and Asia, tick–borne encephalitis. In some cases the illness caused by European subtype of TBEV is asymptomatic. The most often symptoms are fever in the first phase and neurologic symptoms during the second phase. Tick infects itself by virus during co-feeding with infected ticks on the same host but the virus is also transmitted transtadially and transovarially (Gritsun et al. 2003). Viruses occur in tick salivary glands and could enter host in several tens of minutes after the feeding begins.

Castor bean ticks transmit causative agents of human granulocytic ehrlichiosis, human babesiosis and tularemia, diseases with lower occurrence. Human granulocytic anaplasmosis, previously known as human granulocytic ehrlichiosis is caused by *Anaplasma phagocytophilum*, intracellular parasite of human blood cells. The illness has non-specific symptoms (high fever,

headaches, aching muscles and joints) and its development is usually mild; it can cause complications in patients with immunodeficiencies. Protozoan parasites *Babesia microti* or *B. divergens* are causative agents of human babesiosis. This disease has malaria-like symptoms without periodicity. Ticks, as well as some other species, transmit bacterium *Francisella tularensis* causing illness with many symptoms – fever, headache, nausea, ulcerated lesion in the site of tick bite. The majority of detected cases had the origin other than tick bite; for example, pathogen can be transmitted through skin or meat of infected animals (usually hares and rabbits) (Sonenshine et al., 2002; Brouqui et al. 2004).

Castor bean tick is an important vector of animal pathogens. Sheep are endangered by a *Flavivirus* of TBE complex causing louping illness, dogs by canine ehrlichiosis caused by *E. canis* and ruminants by tick-borne fever after infection by *E. phagocytophila*. Different borrelioses might be big problem for multiple animal species: dogs, cats, cattle or horses. Tularemia is a typical disease of hares, rabbits and rodents (Sonenshine et al. 2002; Hillyard 1996).

1.3 ML protein family

ML protein family combines different proteins with ML (MD-2-related lipid recognition) domain. However, their functional characteristics were described only for a few members of this family. Members of this relatively common family were identified in fungi, plants and animals. To date, more than 150 ML proteins are known. This protein family is characterized by presence of a single domain with immunoglobulin (Ig) fold. ML domain carries N-terminal signal peptide sequence and two pairs of cysteine residues at conserved positions (Inohara and Nuñez 2002). Cysteine residues form disulfide bridges that stabilize the tertiary structure of proteins as was confirmed in some members of the family (e.g. Ichikawa et al. 1998; Wright et al. 2000; Friedland et al. 2003). It is also known that some ML proteins are secreted (Kirchhoff et al. 1996; Muellen et al. 2003). Secondary structure is typically formed from β-rich regions joined by multiple strands.

Although the function of the majority of ML proteins has not been identified yet, the ML domain is responsible for lipid binding ability in several proteins from this family. ML proteins are involved in lipid transfer and metabolism or in pathogen recognition and immune response reactions (Inohara and Nuñez 2002).

According to the degree of sequence similarity protein family members are divided into four groups.

1.3.1 Group I

Protein members of the group I of the ML protein family are those most studied. Typical representatives are MD (Myeloid Differentiation) -1 and MD-2 proteins. Both are glycoproteins of 22-25 kDa with two N-glycosylation sites, important for protein functionality, and have no transmembrane regions. MD proteins possess seven cysteine residues in their amino acid sequences, of those only four residues occur in conserved positions. They act in complex with type I transmembrane receptors and carry a domain that is crucial for ligand recognition. Surface expression and activity of receptors depend on MD proteins.

1.3.1.1 MD-2

The MD-2 is a secreted glycoprotein with the length of 160 amino acids and the molecular weight of 25-30 kDa. All orthologs (except for chicken) have seven conserved cysteine residues in their amino acid sequence and two site of N-glycosylation (Visintin 2006) (Fig. 16). MD-2 was first described in human. Then, its orthologs were revealed in many other mammalian species – chimp, macaque, mouse, dog, rabbit, rat, hamster, cow, horse, pig or sheep (Visintin et al. 2006).

MD-2 acts as co-factor of a Toll-like receptor 4 (TLR4); the receptor's ligand is lipopolysaccharide (LPS). TLRs (named due to their homology to the *Drosophila*'s Toll receptor) belong to the group of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), the conserved microbial structures that are almost similar within a group of microorganisms (Medzhitov and Janeway 2000). Examples of PAMPs are LPS, peptidoglycan, synthetic dsRNA, flagellin, CpG motifs associated with bacterial DNA. TLRs are a part of innate immunity where binding of ligand (PAMPs) activates NF-κB and MAP (mitogen-activated protein) kinases and finally leads to the production of cytokines (Kopp and Medzhitov 2003). Structurally, TLRs have three parts: intracellular, transmembrane and extracellular part. The intracellular part contains a TIR (Toll/Interleukin-1 Receptor) domain. The extracellular part carries leucine-rich repeats (LRRs) domain with horseshoe-like shapes that is essential for ligand recognition (Fujimoto et al. 2004).

MD-2 exists in two forms – secreted and bound in complex with TLR4. Only monomeric form of MD-2, which binds to extracellular aminoterminal part of TLR4, interacts with TLR4 (Fujimoto et al. 2004). The residues Arg90, Lys91, Cys95, Asp100, Tyr102, and Cys105 of MD-2 are mostly involved in this process (Re and Strominger 2003). Two regions responsible for interaction with MD-2 exist on the TLR4 molecule: a region with negatively charged residues interacting with positively charged residues on MD-2 (especially with Arg68 and Lys109) and a region with positively charged residues binding negatively charged residues (in a loop between βF strand and α-helix) on MD-2 (Kim et al. 2007). Secreted MD-2 exists as monomers, and also as oligomers, that are linked by disulphide bridges. However, oligomers were observed only

in recombinant MD-2. If native MD-2 also forms multimers is not known (Re and Strominger 2002; Mullen et al. 2003).

Structurally, MD-2 comprises of two β-sheets surrounding a large, narrow, deep hydrophobic cavity with positively charged entrance. The first sheet consists of three antiparallel β-strands; the second sheet has six antiparallel β-strands (Kim et al. 2007; Ohto et al. 2007). The structure is stabilized by three disulphide bridges formed between cysteine residues 25-51, 37-148, and 95-105. Both N-glycosylated residues occur relatively far from the cavity and thus they are, most likely, not involved into ligand binding but into secretion and protection of MD-2 (Ohto et al. 2007).

As was already mentioned, MD-2 in complex with TLR4 acts as co-factor in recognition of LPS. The TLR4-MD-2 complex is formed both in endoplasmatic reticulum and on the cell surface (Visintin et al. 2001; Gangloff and Gay 2004). Similarly to MD-2 interaction with TLR4, LPS is also bound by MD-2 monomer (Re and Strominger 2002; Teghanemt et al. 2008). LPS is bound by serum LPS-binding protein (LBP) that transfers it onto CD14, cell surface molecule. CD14 concentrates LPS and presents it to the MD-2-TLR4 complex (Miyake 2003). LPS or endotoxin occurs in outer membrane of Gram-negative bacteria and consists of three parts: lipid A, core oligosaccharide, and O-side chain. The experiments revealed that MD-2 is responsible for LPS recognition in TLR4-MD-2 complex (Shimazu et al. 1999). As it was shown already, only monomeric MD-2 binds LPS, exactly lipid A, with stoichiometry 1:1 (Viriyakosol et al. 2001). Probably, highly positive regions of MD-2 might be involved in this interaction (Visintin et al. 2003). And how does the LPS recognition occurs? There are two possible mechanisms. The first one: LPS induces linking of two TLR4-MD-2 heterodimers by binding of two different MD-2 molecules through lipid A. The second one: binding of LPS by a TLR4-MD-2 complex enables forming of a heterodimer (Gangloff and Gay 2004). According to last findings, the second model is more probable (Kim et al. 2007; Park et al. 2009).

However, LPS is not the only molecule that is bound by TLR4-MD-2 complex. Plant taxol was also identified as a ligand of the complex (Kawasaki et al. 2003).

Besides LPS binding, MD-2 also influences the amount of TLR4 on the cell surface. For example, soluble MD-2 incubated with HEK293 epithelial cells transfected with TLR4 increases the cell surface levels of the receptor in the absence of LPS (Lauer et al. 2009).

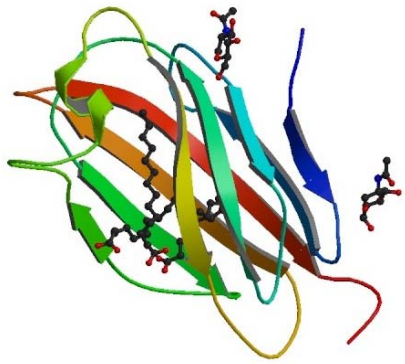

Fig. 3. Tertiary structure of human MD-2 (PDB 2e56).

1.3.1.2 MD-1

MD-1 was discovered by co-precipitation as a molecule associating with RP105 (Radio Protective) receptor on mouse B cells (Miyake et al. 1998). RP105 was described as the mouse B cell surface molecule transmitting an activation signal that leads to resistance against radiation-caused apoptosis and massive proliferation of B cells (Divanovic et al. 2007). Receptor RP105 has extracellular part with LRRs, transmembrane part and a very short cytoplasmic part without TIR domain. It needs the MD-1 ligand that probably binds by N-terminal of LRRs, for its expression on the cell surface. Thus, MD-1 is also expressed on the cell surface (Miura et al. 1998).

The MD-1 is a 162 amino acid long protein with 19 amino acid long N-terminal signal peptide and molecular weight between 22 and 25 kDa (depending on glycosylations).

For the first time MD-1 was isolated from chicken as a *v-myb* regulated gene, but now it is also known in other species – mouse, human, cow and pig (Fig. 15). MD-1 and RP105 are expressed on several types of immune cells with differences in mouse and human (Divanovic et al. 2005). The association of RP105-MD-1 complex with TLR4-MD-2 complex was described. It inhibits TLR4-MD-2 ability to bind LPS and, probably, RP105 acts as a negative regulator of TLR4 mediated LPS responses (Divanovic et al. 2005, 2007; Yoon et al. 2010).

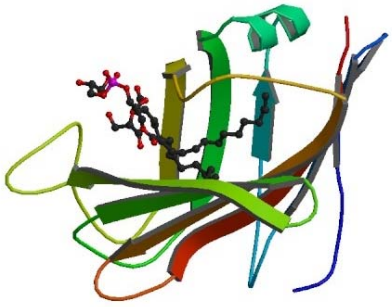

Fig. 4. Tertiary structure of chicken MD-1 (PDB 3mtx).

Recently, the crystal structure of MD-1 was determined. The molecule has β-cup like fold formed from two antiparallel β-sheets. Sheet 1 consists of six β-strands; sheet 2 comprises three β-strands. Three disulphide bridges that connect the two sheets are formed – Cys28-Cys53, Cys40-Cys149, Cys97-Cys107. Sheets surround a hydrophobic cavity with hydrophilic entrance (Yoon et al. 2010). In chicken and mouse orthologs of MD-1 LPS binding was shown. Similarly to MD-2, it binds lipid A but the interaction between MD-1 and LPS precursor lipid IVa is a little different than in MD-2 (Yoon et al. 2010). Probably, MD-1 could regulate LPS sensitivity.

1.3.2 Group II

Group II of ML protein family includes Npc2 and Npc2-like proteins, mite major allergen proteins, 8 *Drosophila melanogaster* and 5 *Caenorhabditis elegans* proteins (Inohara and Nuñez 2002). Three pairs of conserved cysteine residues in ML-domain are characteristic for this group.

1.3.2.1 Npc2 protein

Npc2 (Niemann-Pick protein type C2), firstly identified as HE1 (human epididymal protein 1) protein, is named according to the Niemann-Pick type C disease that is determined by the mutation in genes encoding Npc proteins. (First in late 1920´s A. Niemann and L. Pick described a group of autosomal recessive lysosomal lipid storage disorders with common symptoms; Vanier 2010.) NPC disease is a rare hereditary fatal disorder (with incidence 1:150 000 for Western European population) characterized by defective intracellular transport of endocytosed cholesterol followed by accumulation of free unesterified cholesterol in late endosome and lysosome-like compartments (Patterson 2003; Chang et al. 2005; Huang et al. 2007) leading to progressive neurodegeneration and hepatosplenomegaly (Vanier 2010). The NPC disease is transmitted as autosomal recessive; 95% cases of the disease are caused by mutation of *npc1* gene (encodes transmembrane protein with a putative sterol sensing domain). A mutation in *npc2* gene encoding lysosomal Npc2 protein that binds above all cholesterol is responsible for only 5% of cases (Okamura et al. 1999; Huang et al. 2007). Although two types of disease causes were identified on molecular level, the phenotypic manifestation of both types is the

same (Patterson 2003). To study this disorder an insect *D. melanogaster* model was used (Huang et al. 2007).

Descriprion of glycopeptide called HE1 (later identified as a second gene of NPC disease, Npc2) in human epididymal fluid (Kirchhoff et al. 1996) was the first discovery of ML protein from this group (Npc2-related proteins). Later, it or its homologs presence was described also in other secretory fluids like in bovine milk or human and murine bile (Larsen et al. 1997; Klein et al. 2006) (Fig.17). Human Npc2 protein has 132 amino acids (excluding 19 amino acids of N-terminal signal peptide), forms 3 disulfide bridges and possesses 3 possible N-glycosylation sites from which one site is never glycosylated (Friedland et al. 2003; Chikh et al. 2004; Vanier and Millat 2004; Liou et al. 2006). Microheterogeneity of Npc2 in cells due to variations in glycosylations was revealed in mouse model with npc1 mutation (Chen et al. 2005). Similarly to other soluble lysosomal proteins, Npc2 is likely posttranslationally modified, marked and targeted to endolysosomal system through mannose-6-phosphate receptors (Naureckiene et al. 2000; Willenborg et al. 2005). Here, in late endosomes associated with the internal membranes and lysosomes it acts as cholesterol-binding protein participating in cholesterol homeostasis maintenance (Chen et al. 2005).

If not synthetized endogenously, cholesterol gets into cells by LDL receptor pathway in endocytic vesicles that after obtaining hydrolase enzymes develop into late endosomes and lysosomes (Storch and Xu 2009). Glycoprotein Npc2 is involved in transport and efflux of cholesterol from lysosomes into plasma membrane. Transfer of cholesterol by Npc2 is also influenced by presence of some late endosomal lipids; it is stimulated by bis(monoacylglycerol)phosphate and inhibited by sphingomyelin (Abdul-Hammed et al. 2010). Npc2 binds cholesterol in a 1:1 stoichiometry with binding affinities from 30 nM to 2 μM (Ko et al. 2003; Storch and Xu 2009). Rate of cholesterol transfer from Npc2 to membrane vesicles has been studied. It resulted in finding that Npc2 interacts with membranes by collisional mechanism that is many times faster than an aqueous diffusion-mediated cholesterol transfer. Transfer rates were also higher in a case of membrane with several incorporated anionic phospholipids used or, moreover, at acidic pH or environment with the increased NaCl concentration – up to 2 M (Cheruku et al.

2006; Xu et al. 2008). On egress of cholesterol from lysosomes, Npc2 also cooperates with transmembrane Npc1. Npc1 has soluble N-terminal domain which likely acts as acceptor of cholesterol and thus the protein transfers cholesterol through membrane or through cholesterol transporter. Or, the second possibility is that cholesterol is bound to Npc1, after interaction transfer to Npc2 that transmits the molecule to cholesterol transporter (Infante et al. 2008; Subramanian and Balch 2008).

Another function of Npc2 has been described already. It has been shown that Npc2 binds and tranports the lipid antigen (isoglobotrihexosylceramide) to CD1d molecules (MHC class I-like proteins that present lipids and glycolipids to immune system) in lysosomes during thymocyte selection of NKT (natural killer T) cells. In this process it works as dimer (Schrantz et al. 2007; Liu 2009).

Structural analysis of bovine Npc2 revealed Ig-like β-sandwich fold formed from seven β-strands divided into two β-sheets stabilized by three disulfide bridges (Cys8–Cys121, Cys23–Cys28, and Cys74–Cys80) and three small cavities. The smallest one is connected with a pocket on the surface of the protein (Friedland et al. 2003). The pocket is fully formed only with the presence of bound ligand (Xu et al. 2007). Npc2 is able to bind not only cholesterol, but also other cholesterol-related molecules like precursors of cholesterol biosynthesis – desmosterol, lathosterol; dihydrocholesterol isomers - 5α-cholestan-3β-ol, 5β-cholestan-3β-ol; plant sterols – β-sitosterol, dehydroergosterol, stigmasterol; some 3-positions cholesteryl esters - cholesteryl sulfate, 5α-cholestan-3-one, cholesteryl acetate or an oxysterol - 24-hydroxycholesterol (Friedland et al. 2003; Liou et al. 2006). 3D structure has been determined for single bovine Npc2 (PDB 1NEP) and for bovine Npc2 with cholesterol sulfate complex (PDB 2HKA) (Friedland et al. 2003; Xu et al. 2007).

Fig. 5. Tertiary structure of bovine Npc2 (PDB 1nep).

1.3.2.2 Mite major allergen proteins

Mites are well-known sources of allergens that often cause allergic responses of different severity in human (from allergic rhinitis, asthma to anaphylactic shock). Usually, mite allergens are hidden in house dust, and come from mite feces. They are divided into multiple (19) groups according to their sequence similarity, biochemical and functional characteristics (enzymatic activity, effect on T cells). Group 1 and 2 allergens were identified as the most important allergens. They represent the major part of the mite extracts and show high IgE binding activity *in vitro.* Group 2 allergens are responsible for over 60% of allergies in mite-sensitized people and only they belong to the ML protein family (Thomas et al. 2002; Reginald 2006). Isolated group 2 allergens are summarized in the Table 2 and Fig. 18. These allergens have a high IgE binding activity and as other ML protein family members contain six cysteine residues that form three disulfide bridges in order 1-6, 2-3 and 4-5. However, their exact function is still unknown. The sequence polymorphism was revealed in group 2 allergens as well as in the case of other allergen groups; first in Der f 2 allergen of *Dermatophagoides farinae* (Yuuki et al. 1990). Polymorphism occurs in mites due to the presence of different alleles of the gene. This can affect the recognition of epitopes by T cells, IgE or monoclonal antibodies in allergen molecule (Lagares et al. 2002). Genetic polymorphism was described in group 2 allergens of *D. pteronyssinus*, *D. farinae*, *Glycyphagus domesticus*, and *Lepidoglyphus destructor* among others (Kaiser et al. 2003; Piboonpocanun et al. 2006; Gafvelin et al. 2001; Smith et al. 2001).

Der p 2 and Der f 2 from house dust mites *D. pteronyssinus* and *D. farinae*, respectively, are the most studied allergens from group 2. They show 88% of sequence identity on nucleotide level and 87% on protein level (Mueller et al. 1998; Ichikawa et al. 2005). The tertiary structure of both allergens is stabilized by three disulphide bridges and forms symmetric transient dimers (Ichikawa et al. 1998; Mueller et al. 1998; Rouvinen et al. 2010). Figures 6 and 7 show the tertiary structure of Der p 2 and Der f 2. Recently, a lot of studies were conducted with purpose to determine IgE epitopes or check effect of different

Table 2. Group 2 allergens of mites.

Organism	Name	Accession number	Number of amino acids	Molecular weight (Da)	References
house dust mites					
Dermatophagoides pteronyssinus	Der p 2	AAF86462	145	15867	Chua et al. 1990
Dermatophagoides farinae	Der f 2	BAA01240	146	15802	Yuuki et al. 1991
Euroglyphus maynei	Eur m 2	AAC82349	145	15746	Smith et al. 1999
storage mites					
Tyrophagus putrescentiae	Tyr p 2	O02380	141	14851	Eriksson et al. 1998
Psoroptes ovis	Pso o 2	Q965E2	143	15211	Temeyer et al. 2002
Lepidoglyphus destructor	Lep d 2	CAA61419	141	14772	Schmidt et al. 1995
Glycyphagus domesticus	Gly d 2	CAB76459	123	13179	Gafvelin et al. 2001
Aleuroglyphus ovatus	Ale o 2	AAS75832	144	15531	Ramjan and Chew 2003
Suidasia medanensi	Sui m 2	AAS75831	141	15071	Reginald et al. 2006
Blomia tropicalis	Blo t 2	ABG76185	142	15179	Reginald et al. 2006

mutations on the allergenicity (Nishiyama et al. 1995; Takai et al. 1997, 2000, 2001; Nakazawa et al. 2005).

Der p 2 is a 14 kDa protein with homology to Npc2 protein (among allergens Der f 2 and Lep d 2). It is formed by two antiparallel β-sheets (sheet 1 contains strands from residues 13-17, 34-42 and 85-93; sheet 2 comprises residues 6-8, 51-58, 61-64, 104-112 and 115-122) that overlay each other at an angle of approximately 30°. However, two strands were not defined by NMR

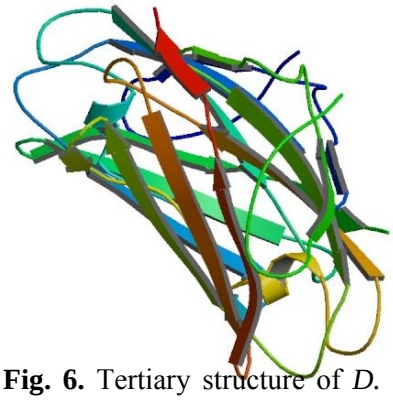

Fig. 6. Tertiary structure of *D. pteronyssinus* allergen Der p 2 (PDB 1ktj).

spectroscopy but only by crystalography (Mueller et al. 1997, 1998; Derewenda et al. 2002). Remaining residues create loops and also a short α-helix (residues 72-75). Because some regions of the allergen were described in different conformations, it is possible that they move slowly (Mueller et al. 1997, 1998). Structure forms an internal cavity, covered with hydrophobic and aromatic amino acid residues that leads on the surface to a short tunnel rich in proline residues. Der p 2 is considered to be a lipid-binding protein without enzymatic activity; this theory is supported by the presence of the hydrophobic residues in the cavity (Derewenda et al. 2002). Several experiments have been made to determine the ligand of the allergen but all of them brought no results. Also LPS was tested and it was observed that Der p 2 binds it although with a very low affinity (Manček Keber et al. 2005). On the other hand, Der p 2 was shown to mimic MD-2 by facilitating LPS signaling through binding not only by LPS, but also by TLR4 (Trompette et al. 2009). The IgE epitopes of the allergen are conformational, stabilized by disulphide bridges that are crucial for antibody recognition. Several amino acid residues (44-46, 100) on the molecule surface are involved in recognition by IgE (Smith and Chapman 1997; Mueller et al. 1998). Besides, studies directed on localization of regions responsible for recognition of Der p 2 by T cells in the context of MHC (major histocompatibility complex) class II were performed. For example, regions of amino acid residues 25-31, 78-104, or 87-112 were determined as important for recognition by T cells and stimulating their proliferation in mice (Wu et al. 2002; Hoyne et al. 1993), while peptide 111-129 was mostly recognized by human T cells (O'Brien et al. 1995). Although the function of Der p 2 is not known yet, the site in *D. pteronyssinus* body, where allergen

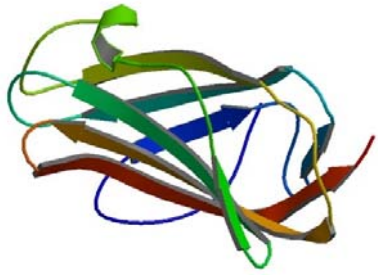

Fig. 7. Tertiary structure of *D. farinae* allergen Der f 2 (PDB 2f08).

occurs, was revealed. In both sexes of the mite, it was localized in the gut (epithelial cells) and in the fecal pellets (Park et al. 2000).

The other most studied mite group 2 allergen is Der f 2 from *D. farinae*. Like Der p 2 it is also 14 kDa molecule with a high degree of similarity to the *D. pteronyssinus* allergen. Nevertheless, their crystal structures are different. Der f 2 is formed from two major anti-parallel β-sheets (sheet 1 consists of strands from residues 13-18, 34-43, and 85-93; sheet 2 contains strands 5-7, 51-58, 61-63, 104-112, and 115-122), an additional small β-sheet (residues 27-30 and 125-128) and a short α-helix (formed from residues 71-75) (Ichikawa et al. 1998, 2005; Johanessen et al. 2005). The two major β-sheets overlay each other at an angle of approximately 30° (Ichikawa et al. 2005; Suzuki et al. 2006). Der f 2 carries two cavities: a larger cavity that is covered by hydrophobic and aromatic amino acid residues and is joined with exterior by a tunnel, and a smaller cavity near the mouth of the tunnel (Ichikawa et al. 2005; Johanessen et al. 2005). Many studies focused on determination of IgE epitopes on the allergen molecule, or on effect of different mutations of Der f 2 on IgE binding have been made. It was revealed that two disulphide bonds (8-119, 73-78) and the surrounding regions are important for recognition by IgE (Nishiyama et al. 1995). Observation of allergen binding of *Escherichia coli* lysate was published (Ichikawa et al. 1998). Moreover, Der f 2 allergen binding to LPS in nanomolar affinity and 1:1 molar ratio, that could be inhibited by Ca^{2+}, was also revealed (Ichikawa et al. 2009). Probably, Der f 2 could act as a component in antibacterial defense processes.

1.3.3 Group III

Not many proteins from group III of ML protein family have been discovered yet. Closer described and the most studied member of this group is a phospholipid transfer protein of *Aspergillus oryzae*, phosphatidylglycerol/phosphatidylinositol transfer protein (PG/PI-TP). Its putative orthologs and paralogs belong to this group as well as at least nine *Arabidopsis thaliana* proteins (Inohara and Nuñez 2002) (Fig.19).

1.3.3.1 PG/PI-TP of *A. oryzae*

The cDNA of the oligospecific phospholipid transfer protein, isolated from filamentous fungus *A. oryzae*, is encoded by a single gene that is translated into a 175 amino acids protein (Record et al. 2001). The amino acid chain carries the leader sequence composed of the putative signal peptide (1-21 aa) and the extra N-terminal signal sequence (22-37 aa) that is followed by a 30 amino acid long N-terminal sequence revealed in purified PG/PI-TP. A putative transmembrane domain (5-29 aa) is predicted in the site of the leader peptide. These parts of N-terminal sequence indicate that PG/PI-TP may be a secreted protein (Record et al. 1999). The mature protein is 138 amino acids long and has molecular weight of approximately 15 kDa. The PG/PI-TP expression seems to be regulated at the mRNA level. Its expression in fungus is not limited by presence of phospholipids in culture medium with glucose (Record et al. 1999). In the cells, the transfer protein was localized in the membrane of Golgi-like secretory vesicles and also in cytoplasm (Record et al. 1998).

1.3.4 Group IV

The most studied member of the group IV of the ML protein family is GM2-activator protein (GM2-AP) that has defined crystal structure. Other members of the group IV are GM2-AP orthologs as well as CheB proteins of *D. melanogaster* (Inohara and Nuñez 2002; Starostina et al. 2009) (Fig 20). These proteins are involved in perception of pheromones. They are secreted into lumen of pheromone-sensing hairs where they probably interact directly with lipid-like pheromones and modulate their detection (Starostina et al. 2009).

1.3.4.1 GM2-activator protein

GM2-AP is a small lysosomal or secreted protein with important roles in lipids metabolism and T cell activation. It binds, solubilizes and transports a variety of lipids (glycolipids, gangliosides, phosphoacylglycerol). However, it has and plays an essential role in hydrolysis of ganglioside GM2 to GM3 catalyzed by β-hexosaminidase A (Hex A). Mutation in a *gm2-ap* gene results in AB variant of GM2 gangliosidosis, fatal disease that is characterized by accumulation of GM2 primarily in neuronal cells. Patients suffer from progressive motor weakness, neuromuscular deterioration, exaggerated startle

response to sharp sounds, have a sherry red spot in the fundus of the retina, in later stages of the disease, macrocephaly, seizures, dysphagia, and die before four years of age (Martin et al. 2005). This disease is one of three diseases that are characterized by GM2 accumulation; the further two illnesses are Tay-Sachs and Sandhoff disease that are caused by mutations in genes encoding α or β-subunit of Hex A.

GM2-AP is synthetized in rough endoplasmatic reticulum as 193 amino acid long prepolypeptide with 23 amino acid long signal peptide. It is usually glycosylated in one N-glycosylation site. The forms of propolypeptide differ in their molecular weights according to the type of glycosylation: 22 kDa form has a highly-mannose type and 24-27 kDa has a complex type, a part of molecules stay non-glycosylated. GM2-AP molecules with mannose type oligosaccharides can be marked by phosphate groups that protect oligosaccharide before further modifications in Golgi. A portion of such marked protein is secreted; the other part is targeted to the endosomal-lysosomal pathway through interaction with mannose-6-phosphate receptor (MPR). Activator molecules can be recaptured to lysosomes by MPR-dependent and MPR-independent pathway, which is saccharide-independent (Rigat et al. 1997). The further processing of N-terminus occurs in lysosome and results in a 162 amino acid long (17 531 Da) mature protein (Mahuran 1998).

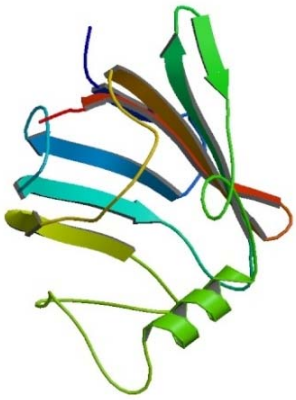

Fig. 8. Tertiary structure of human GM2-AP (PDB 1g13).

Crystal structure of GM2-AP was determined for activator alone and also in complex with bound lipids (Wright et al. 2000, 2003, 2004). Activator is folded into eight-stranded cup-shaped anti-parallel β-plated sheet. It consists of six nine-to-ten residue β-strands, an interrupted strand and a short strand; four disulphide bridges stabilize protein structure. Central hydrophobic cavity is not buried and is accessible for lipids. The cavity's mouth is splitted from the surface by a rim formed from flexible loops and short α-helix. It was revealed that these loops and residues (residues 125-133, Trp131)

control entry of lipid molecules into the cavity and exist in different conformations – open and closed – that are stabilized by hydrogen bonding network (Wright et al. 2000, 2003).

A basic function of activator is its ability to bind and solubilize a wide spectrum of lipids, mainly acidic glycosphingolipids and gangliosides, acting as a biological detergent. It can transport them among liposomes. The transport is faster in environment with pH 5 than in pH 7, as it was observed *in vitro* (Mahuran 1998). GM-2 AP recognizes mostly negative-charged residues on glycosphingolipids or sulphate groups of gangliosides (Hama et al. 1997).

During GM2 hydrolysis the activator plays a crucial role in interaction with ganglioside GM2 and Hex A. It binds and solubilizes GM2 by partial inserting into the lipid bilayer, lifting GM2 out, and presenting trisaccharide moiety in soluble form to Hex A that cleaves *N*-acetylgalactosamine (GalNAc) (Wright et al. 2000). Activator's residues Trp131, Trp63 participate, probably, in insertion into the lipid bilayer (Wright et al. 2003). Thus, activator must have regions involved in GM2 and Hex A recognition and binding. GM2-AP binds GM2 at two sites: within N-terminal part (residues 32-142) there is sialic acid binding site, and within C-terminal part (residues 143-193, especially Asp156, Glu158) GalNAc binding site occurs where activator presents GalNAc to Hex A (Wright et al 2000). Probably, the α-helical region of activator (Phe111-Pro120) participates in the interaction with Hex A, especially with its flexible loops in α-subunit (280-283, 396-398) (Zarghooni et al. 2004; Wendeler et al. 2006). Residues Asn423, Arg424 of the α-subunit of Hex A are responsible for GM2 binding (Lemieux et al. 2006).

Besides GalNAc hydrolysis of GM2 to GM3 by Hex A, activator also stimulates other catalytic reactions. They are: sialic acid hydrolysis of GM2 to GA2 by clostridial sialidase, hydrolysis of GalNAc from dipalmitoylphosphatidylethanolamine- $I1^3NeuAcGgOs_3$ by Hex A, liberation of galactose from GM1 by β-galactosidase, and ADP ribosylation by phospholipase D (Wu et al. 1994; Nakamura et al. 1998).

1.4 ML proteins in ticks

Although proteins belonging to ML protein family occur relatively often in many organisms, data about their presence, distribution and functional activities in ticks are very limited. A list of identified ML proteins in ticks is presented at Fig. 21 (Appendix). ML proteins were described in soft ticks *Ornithodoros coriaceus*, *O. parkeri* and in hard ticks *Rhipicephalus microplus*, *R. appendiculatus*, *Dermacentor variabilis*, *Ixodes scapularis*, and *I. ricinus* (Francischetti et al. 2009). The sites of their expression include several tissues. Analysis of midgut transcripts of *D. variabilis* revealed that transcripts of the genes encoding ML domain containing proteins are the third most abundant transcripts (specifically those of allergen-like proteins) in this tick species (Anderson et al. 2008). Transcripts of ML protein genes were detected in the midgut of *R. microplus* (Kongsuwan et al. 2010), in salivary glands of soft tick *O. parkeri* (Francischetti et al. 2008), and in male accessory glands/testis/vas deferens of *D. variabilis* fed males (Sonenshine et al. 2011) and in the body of *I. scapularis* (de la Fuente et al. 2008). All identified transcripts can be assigned to the group II of ML protein family. Three genes encoding proteins from ML family were found in *O. parkeri* salivary glands (Francischetti et al. 2008). At the same time five genes encoding ML proteins in *D. variabilis* midgut were detected: three of them are defined as allergen-like related; the other two are Npc2-related. All of them are probably involved in tick immunity. Two transcripts were found only in partially fed ticks (2 days fed), while three other occurred both in unfed and in (partially) fed (2 and/or 6 days fed) ticks (Anderson et al. 2008). In conclusion, ML proteins are constitutively expressed in tick body and their expression is also induced by blood meal.

In *I. ricinus*, four genes encoding ML domain containing proteins were identified. They are: alerg1 - gene for allergen-like protein (AJ547805), gene encoding Der-p2 allergen-like protein (EF116564), and two genes encoding two different ML domain-containing proteins (AY323234, EU034645) (Jacot 2003; Rudenko et al. 2005; Horáčková et al. 2010a, 2010b). The first described ML domain-containing protein was identified only in tick's gut. Its gene expression was induced by blood feeding or *B. burgdorferi* infection (Rudenko et al. 2005). The 116 amino acids long allergen-like protein was also found in

midgut. Blood feeding induces its expression that occurs between the second and the sixth day of feeding. Allergen-like protein was localized only in secretory vesicles of secretory cells in the gut between day 1 and 6 of tick feeding (Jacot et al. 2003).

Briefly, the genes encoding members of ML protein family were identified in different tissues of hard and soft ticks. The most frequent site of expression is midgut. Their expression is, in generally, induced by blood feeding. However, several members of tick ML protein family were expressed also in unfed ticks. Probably, proteins can be secreted from secretory cells of tissues into the lumen of organs or hemocoel and can act as transport or immune-related molecules.

This work is dedicated to ML-domain containing protein (ABU43149) and Der-p2 allergen-like protein (ABL61513) from castor bean tick *Ixodes ricinus*, the main vector of multiple infectious diseases of humans in Europe.

2. Objectives

The main aim of the work was to identify, isolate and characterize genes encoding proteins from ML domain protein family in the castor bean tick *I.* ricinus. Our attention was focused on structural and functional analysis of recombinant ML-domain containing protein and Der-p2 allergen-like protein. Being involved in transport and metabolism of lipids or in recognition of pathogen components such as LPS, tick ML proteins might be participants of tick immune response to infection or mediators of defensive reaction. Tick ML proteins might probably cooperate with the other tick proteins in pathogen recognition or in the process of digestion.

Specific aims were:

- Identification and isolation of the genes encoding ML proteins in *I. ricinus* and characterization of them by bioinformatics tools. Focusing on finding of characteristic features of tick ML domain (number of disulphide bridges, their sites, presence of N-terminal signal peptide, etc.), comparison with other related ML proteins; determination of amino acids important for possible lipid binding ability.
- Determination of expression patterns of both genes: time-, stage-, and tissue-specific expression; comparison of the gut- and salivary gland-expressed ML proteins of *I. ricinus* with respect of their possible role in tick immune response.
- Localization of the gene expression directly in dissected tick tissues and specification of the site of expression.
- ML proteins are reffered as immune-related proteins. Our aim is to analyse the possible roles of identified ML proteins in tick innate immunity and their interaction with different microorganisms. Determination of antimicrobial activity of recombinant tick ML proteins or their microbial-products binding ability is one of our goals.
- Determination of possible IgE binding ability, characteristic for allergens, especially in Der-p2 allergen-like protein.

3. Results

3.1 ML-domain containing protein (ABU43149)

Two genes encoding ML proteins were identified in *I. ricinus* and named accordingly to the content of the characteristic domain. Part of this work is dedicated to the ML-domain containing protein (IrML), described as the second one. The gene of IrML (EU034645) was identified due to its high sequence similarity to the partial mRNA sequence of ML domain-containing protein (AY323234) described before.

The existence of several polymorphic forms of IrML was revealed during the screening of subtracted cDNA library from the whole body of *I. ricinus*. Nucleotide substitutions identified in individual recombinant clones led to appearance of 8 different protein isoforms. Table 3 below shows the positions with changed amino acids. The most frequent were changes of amino acids with hydrophobic side chain (Lys, Met) by another one (Met, Lys, Ala) and hydrophobic amino acid (Lys, Ala) by polar uncharged amino acid (Thr). The highest number of amino acids substitutions detected in comparison with primarily identified IrML (ABU43149) was five.

Table 3. Polymorphism in IrML.

Position nr.	Reference - IrML (ABU43149)	Amino acid changes
4	Met	Leu/Glu
8	Ala	Leu
18	Ala	Thr
38	Met	Thr
46	Leu	Met
69	Ile	Val
72	Arg	Gly
87	Glu	Pro
96	Lys	Arg
97	Ser	Asn
102	Asn	Glu
103	Met	Ala
116	Leu	Thr

IrML contains an ML domain that places it into the group II of ML protein family (together with Npc2-like or allergen-like proteins). As for the rest of protein members of group II, a possible ability of IrML to interact with lipids is predicted by the presence of putative lipid binding site, formed by residues 30, 41, 44, 46, 56, 62, 64, 66, 79, 81, 83, 100, 110, 118, 134, 136, 138, 140, 146, 149, 151, 153 (Marchler-Bauer et al. 2011). The presence of predicted N-terminal signal peptide defines the IrML as secretory pathway signal peptide. The predicted tertiary structure of IrML is highly similar to it of Npc2 protein from members of ML protein family with the known crystal structure. Both molecules consist of two β-sheets composed of three and four strands (Fig. 9). Essential three disulphide bridges are formed in the same order. Contrary to Npc2, IrML molecule is glycosylated in *I. ricinus*.

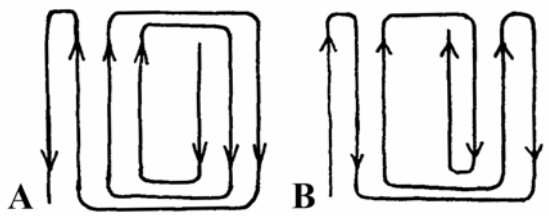

Fig. 9. Scheme of IrML (A) and Npc2 (B) folds.

It was shown earlier that gene expression of ML domain-containing protein was induced by blood feeding or *B. burgdorferi* infection (Rudenko et al. 2005). Similarly to gene expression of ML domain-containing protein, IrML gene expression is also influenced by blood feeding of the tick. IrML gene expression, determined by analysis of whole body RNA, starts after beginning of feeding, fluently increases to the third day of feeding and stays approximately on the same level. IrML was localized in gut, hemolymph and salivary glands of *I. ricinus*. However, the highest expression level occurred in the tick gut. The time expression of *IrML* was compared between gut and salivary glands. Interestingly, the gene expression in the gut repeated the expression observed in the whole body but the expression found in salivary glands was different. It started after the third day of feeding and showed the permanent increasing. The same dynamics of gene expression was confirmed by *in situ* hybridization (ISH) technique. Tissues of unfed ticks did not show any positive signal; the positive staining was detected in partially fed ticks. Localization was more successful in salivary glands where *IrML* was expressed

mainly in type II and III acini. Unfortunately, analysis of the gene expression in the tick gut by ISH technique did not show reliable results due to the high nonspecific signal.

Although specific polyclonal antibodies against recombinant IrML were prepared, detection of native IrML in tick was not succesful (by imunodetection, or immunoprecipitation).

Because the members of ML protein family are known to be involved in innate immune processes, we examined the IrML effect on vitality of different microorganisms. We also checked the ability of selected microorganisms to affect the *IrML* gene expression. The experiment on the analysis of impact of different microorganisms on *IrML* gene expression was performed on *I. ricinus* embryonal cell line IRE/CTVM 19. Tick cell line was incubated with selected inactivated microorganisms: *Micrococcus luteus*, *Escherichia coli*, *B. burgdorferi* and *Candida albicans*. As it is shown on Figure 11, the presence of *M. luteus* or *C. albicans* in a tick culture didn't show any affect on *IrML* expression in comparison to tick cells alone. Contrary to this, the presence of *E. coli* and *B. burgdorferi* significantly induced expression of *IrML* gene. Revealed results confirm the involvement of *I. ricinus* IrML in recognition of pathogen related compound. Gram-negative bacteria (*E. coli, B. burgdorferi*) upregulate the *IrML* expression in embryonal cell line IRE/CTVM 19. The opposite impact of recombinant IrML on vitality of selected microorganisms' was not confirmed. Neither incubation of bacteria (*M. luteus*, *Staphylococcus xylosus*, *E. coli*, *Pseudomonas fluorescens*, *Enterobacter cloacae*, *C. indologenes*) with IrML in liquid medium nor application of IrML into the solid medium on Petri dishes showed any effect on bacteria survival. Most probably *I. ricinus* IrML is involved in pathogen recognition, but not in elimination of invader in the tick body alone. It is possible that increased expression of *IrML* in response to pathogen invasion might trigger the activity of synergetic molecule that will start the defense reaction. IrML may stimulate other mechanisms in tick immune response and participate in other defense pathway.

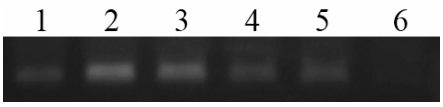

Fig. 10. Expression of *IrML* in tick cell line incubated with microorganisms. 1 – *M. luteus*, 2 – *E. coli*, 3 – *B. burgdorferi*, 4 – *C. albicans*, 5 – tick cells alone, 6 – negative control.

3.1.1 Research article I

IrML - a gene encoding a new member of the ML protein family from the hard tick, *Ixodes ricinus*.

Horáčková, J., Rudenko, N., Golovchenko, M., Havlíková, S., Grubhoffer, L.
J. Vector Ecol. 35 (2010), 410-418.

Abstract

Blood intake causes significant changes in ticks, triggering vital physiological processes including differential gene expression. A gene encoding *Ixodes ricinus* ML-domain containing protein (IrML) is one of the set of the genes that are strongly induced by blood meals. IrML belongs to the ML protein family that commonly occurs in diverse organisms and is involved in lipid binding and transport, pathogen recognition or in immune response. An *IrML* gene was amplified from cDNA of engorged *I. ricinus* females using the gene-specific primers designed on a basis of partial sequences of related genes for ML domain protein. *IrML* was shown to be expressed mainly in the gut, but also in salivary glands and hemolymph of all tick developmental stages. Using in situ hybridization, IrML transcripts were detected in type II and III salivary glands acini. Analysis of the predicted structure of *I. ricinus* ML-domain containing protein and its localization in the tick body could suggest that IrML is a secreted protein and is possibly involved in tick innate immunity.

3.2 Der-p2 allergen-like protein (ABL61513)

Der-p2 allergen-like protein (D-Al protein) of *I. ricinus* was isolated during experiments with allergen-like protein (AJ547805). Two mentioned proteins showed highly similar N-terminal part but differ in the C-end of the molecule as is shown in Figure 12. Allergen like protein has 116 amino acids, while D-Al protein has 144 amino acids. Both proteins contain 16 amino acid long N-terminal peptide and Der-p 2-like domain, present in group II members of ML protein family. A putative lipid binding cavity in Der-p2 allergen-like protein is predicted (residues 23, 32, 35, 37, 47, 53, 55, 57, 70, 72, 74, 90, 99, 107, 122, 124, 126, 128, 134, 137). *D. farinae* allergen Der f 2 is the molecule with defined tertiary structure that showed the highest homology to *I. ricinus* D-Al protein. Both molecules form a structure with two main sheets comprised of three and four strands (Fig. 12). No predicted glycosylated residues were detected.

```
ABL61513    1 MFRYLVFLLIVGAVSCQRRKAVYKPCGGSGKLISVEVEPCDSDPCVFKKGTDVKVHVTMV
CAD68004    1 MFRYVVFLLIVSAVSCQRRKAVYKPCGGSGKLISVEVGPCDSDPCVFKMGTDAKIHVTMV

ABL61513   61 ADQDSDTATLDARVKVFGFQMPVPGIETDLCKGTVECPVIKGRKYSVTAIFPVPSLMSLK
CAD68004   61 ADQDSDTATLDARVKVFGFQMPVPGIETDLCKGTVDCPVIRD----GSTASPPSSLCPLS

ABL61513  121 TEVTFKVIGDKGLSVCGQSDIVIE
CAD68004  117 ------------------------
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Fig. 11. Alignment of Der-p2 allergen-like protein (ABL61513) and allergen-like protein (CAD68004) of *I. ricinus*. Predicted signal peptide is written in italics.

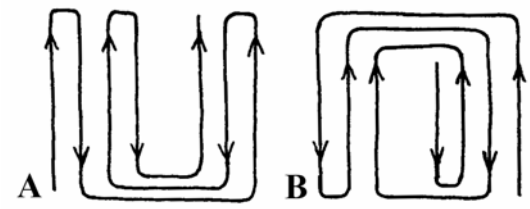

Fig. 12. Scheme of D-Al protein (A) and Der f 2 (B) folds.

Gene encoding D-Al protein is not expressed in unfed adult ticks. The other tick developmental stages showed just a basic level of its expression. Expression of *D-Al* is induced by blood intake. It starts on the first day of feeding and reaches the maximum level on the third day of blood intake. Analysis of gene encoding D-Al protein expression in tissues (gut, salivary glands, hemolymph, ovaries, Malphigian tubes) revealed that it is mainly gut-specific, but it is also detected

in hemolymph. *In situ* hybridization technique did not reveal localization of mRNA in the gut. Specific antibodies against D-Al protein were prepared, but isolation of native tick molecule was not successful.

The exact role of allergens, especially of group 2 allergens, is not known even in mites, although they are the typical representatives of organisms producing highly allergenic proteins. However, Der f 2 of house dust mite *D. farinae* is proposed to be involved in its innate immunity. The *in vitro* binding assay of Der f 2 showed its ability to bind *E. coli* (Ichikawa et al. 1998). Analogous experiments with recombinant tick D-Al protein resulted in interesting findings. The tick protein binds to *E. coli* but also, in lower degree, to *M. luteus* (Fig. 13). However, analysis of antimicrobial activity of D-Al protein did not give any results indicating that it affects the bacterial growth or it is involved in bacterial killing. Thus, D-Al protein can probably bind some compound of *E. coli* (or another bacterium) when it gets into the tick's body. Maybe, it can interact with bacterial molecule and "presents" it for further processing.

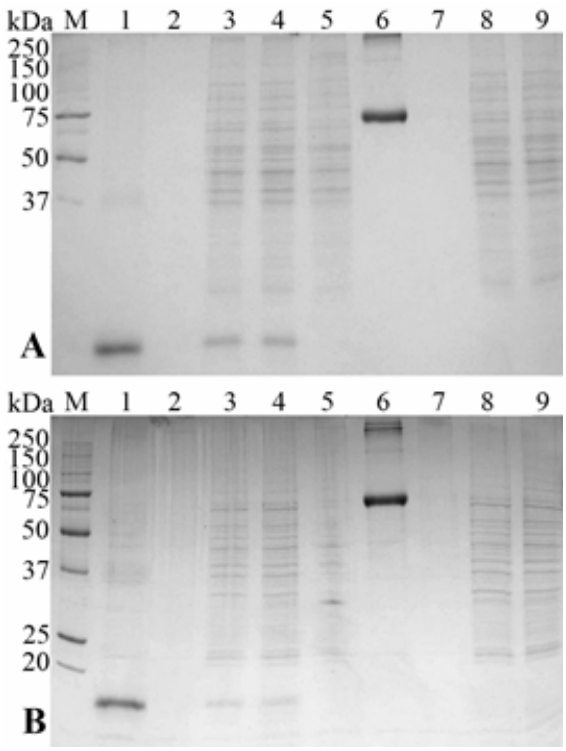

Fig. 13. *In vitro* binding assay of D-Al protein and *E. coli* (A) or *M. luteus* (B). 1 - purified D-Al protein, 5 – *E. coli* or *M. luteus*, 6 – BSA, 2+7 – wash fraction, 3+8 – 3 min incubation, 4+9 – 30 min incubation.

Another experiment directed on the analysis of impact of selected microorganisms (*M. luteus*, *E. coli*, *B. burgdorferi*, *C. albicans)* on expression of *D-Al* in tick cell line IRE/CTMV 19 did not bring any positive results. None of the microorganisms involved in analysis induced expression of the gene encoding D-Al protein in tick cells.

Although *I. ricinus* bite usually does not cause the allergic reaction or even anaphylactic shock, we examined if D-Al protein is recognized by human immunoglobulin E antibodies. We found that the tick D-Al really can bind IgE. That indicates that it also can be distinguished by IgE. Maybe, D-Al protein can neutralize antibodies and protect tick gut during blood intake and digestion. Or, it can be bound by antibodies due to its similarity (probably partial) to molecule(s) recognized by these antibodies.

3.2.1 Research article II

Der-p2 (*Dermatophagoides pteronyssinus*) allergen-like protein from the hard tick *Ixodes ricinus* - a novel member of ML (MD-2-related lipid-recognition) domain protein family.

Horáčková, J., Rudenko, N., Golovchenko, M., Grubhoffer, L.
Parasitology. 137 (2010), 1139-1149.

Abstract

OBJECTIVE:
Expression of the gene encoding Der-p2 allergen-like protein in the castor bean tick *Ixodes ricinus* is induced by blood intake. Tick Der-p2 allergen-like protein belongs to a diverse family of ML proteins that includes major allergens of house dust mites, human MD-2 or similar proteins from *Drosophila melanogaster*. In ticks, genes encoding proteins belonging to the ML protein family were identified, but their protein products have not been characterized yet.
METHODS:
A gene encoding tick Der-p2 allergen-like protein was amplified from cDNA of engorged *I. ricinus* female using the gene-specific primers designed on a basis of partial sequences of related allergen-like genes. The tissue and state specific patterns of expression of the gene were analysed. The IgE binding activity of the produced recombinant protein was studied by use of ELISA.
RESULTS:
Analysis of the expression pattern showed that the gene encoding the tick Der-p2 allergen-like protein is strongly induced by the blood meal in gut and haemolymph throughout all tick developmental stages. Der-p2 allergen-like protein possesses a putative lipid-binding site, according to the comparisons with the related proteins. The ability of tick Der-p2 allergen-like protein to bind immunoglobulin E (IgE) was revealed.

DISCUSSION:

The presence of a putative lipid-binding domain in Der-p2 allergen-like protein and its ability to interact with IgE might indicate the involvement of the protein in the tick’s immune response.

4. Discussion

Our knowledge about the representatives of invertebrate ML proteins family is very limited. Identification and characterization of two novel members of ML protein family of castor bean tick *Ixodes ricinus* was the main aim of this work.

Sequence analysis of *I. ricinus* ML-domain containing protein revealed its high similarity to several predicted or hypothetical proteins of different tick species. The highest homology was shown to the putative ML domain-containing protein from *I. scapularis* (XP_002434499; identity 94%, similarity 97%) and the putative ML-domain containing protein from *Dermacentor variabilis* (ACF35538; id. 62%, sim. 75%). However, the closest relative to IrML was confirmed to be Sui m 2 of mite *Suidasia medanensi* (AAS75831; id. 28%, sim. 42%). Other related proteins represent Npc2 or Npc2-like proteins of different organisms with identity between 25 and 35% and similarity from 45 to 55% confirming that *I. ricinus* IrML belongs to Npc2-like group of ML protein family and also partially to lipid tranfer proteins.

IrML is expressed during blood feeding with increasing pattern first in the gut, then in hemolymph and with delay in salivary glands (during the second half of feeding). This fact leads to the presumption that IrML plays an important role in tick gut. As it is known, molecules involved in blood digestion and metabolism (enzymes and their inhibitors, ferritin), immune mechanisms (allergen-like, or lectin-like proteins), molecules associated with oxidative stress (glutathione-S-transferase, thioredoxin), cell, lipid and carbohydrate binding molecules (mucins, glycine rich proteins) are primarily expressed in the gut of ticks during the blood meal (Anderson et al. 2008). The first two mentioned groups are represented in *I. ricinus* mainly by a network of digestive peptidases (Horn et al. 2009) for the first group and, Ixoderin A, a fibrinogen-related protein, α2-macroglobulin, defensins for the second group (Rego et al. 2005; Rudenko et al. 2005, 2007; Burešová et al. 2009; Chrudimská et al. 2011). Some of the studied molecules, mostly involved in tick immune response, are also expressed in other *I. ricinus* tissues, mainly in hemolymph and salivary glands (Chrudimská et al. 2011). Hemolymph, surrounding all internal organs and tissues, among other molecules contains different proteins

and peptides – transfer proteins, vitellogenin, lectins, antimicrobial proteins, and others (Gudderra et al. 2002). Compounds obtained from blood, digested or undigested in the gut, are transported free to target tissues through hemolymph or bound to carrier proteins. It is possible that IrML is transfered from gut into the hemolymph as well. Because the detection of native IrML was not succesful yet, it would be interesting to find out exactly where it occurs during the process of feeding and if it really is distributed from gut through hemolymph into other tissues or if its localization remains unchanged (focusing on site and time). Whereas in salivary glands the *IrML* expression starts during the second half of feeding - rather at the end of slow feeding period of the tick, probably it deals with the independent expression compared to the gut-expressed *IrML*. The salivary glands' IrML might have different function than the gut specific. The IrML probable site of action is lumen of organs or hemocoel – in salivary glands it is produced by secretory cells, in hemolymph by hemocytes, and it is possibly also secreted in the gut.

Since there is almost no information about functions of ML proteins (from group II), especially in invertebrates, we can only speculate about the processes and mechanisms in which IrML might be involved. Two most probable functions come into consideration. First, IrML is involved in tick innate immunity; second, it serves as a transport molecule. The experiments with IrML effect on microorganisms showed that it has not direct impact on their growth or survival but *IrML* expression is influenced by Gram-negative bacteria. These observations indicate that it could be involved in immune response to Gram-negative bacteria and in direct binding of specific bacterial molecule in particular, and serves as soluble PRR. Or it could be involved in interaction and transport of a bacterial component, or as a regular participant in immune defense process against ingested bacteria. Nevertheless, there is a possibility that IrML binds with different degree of specificity lipidic molecules and transfers them from the gut to the target tissues.

As IrML, the Der-p 2-like allergen protein sequence showed the highest similarity to hypothetical proteins of different ticks (besides *I. ricinus* allergen like protein). The most similar were the hypothetical protein of *Amblyomma maculatum* (AEO32826; id. 46%, sim. 72%) and the conserved hypothetical protein of *I. scapularis* (XP 002400837); id. 47%, sim. 41%). But the allergen

Pso o 2 of *Psoroptes ovis* mite (Q965E2) with the identity of 36% and the similarity of 58% is the most related one from the described proteins. Then follow the allergens of different mite species with identities and similarities ranging from 30 to 35% and 50-58%, respectively.

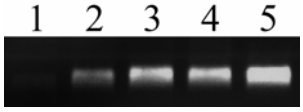

Fig. 14. Time expression of *D-Al* in *I. ricinus* females. 1 – unfed ticks, 2 – 1-day fed, 3 – 3-day fed, 4 – 5-day fed, 5 – 7-day fed ticks.

Expression of gene encoding D-Al protein starts after blood intake and after the first day of feeding stays practically on the same level (Fig. 14). Besides hemolymph, midgut was defined as another site of the gene expression. Belonging of D-Al protein to ML protein family, its localization and other features suggest its involvement into transport mechanisms or tick immunity. The majority of known mite group 2 allergens occur in the gut. However, similar molecules were found in ticks salivary glands also.

Experiments for revelation of D-Al protein impact on bacterial growth, as decibed above, were performed. Adding of recombinant D-Al protein into the liquid or solid medium with different microorganisms did not affect their growth or vitality in any way. However, the *in vitro* binding assay conducted according to Ichikawa et al. (1998) showed that D-Al protein binds *E. coli* (Fig. 14). This result indicates the capacity of D-Al protein to bind to selected Gram-negative-specific molecules. Ichikawa et al. (2009) found out that Der f 2 binding of *E. coli* molecule was most probably caused by LPS binding. Possible LPS binding was not tested in D-Al protein due to low content of LPS in the sample.

Besides bacterial binding, D-Al protein has other binding ability – immunoglobulin E binding. IgE recognition and binding is one of the characteristic features of common allergens. However, ticks are not typical organisms that cause the allergic reaction neither to tick bite nor to their products (of course with some exceptions). But what is the reason for D-Al protein to bind IgE? Possibly, IgE that gets into the tick body with bloodmeal could be “neutralized” by D-Al protein. IgE can be transfered then through hemolymph into the salivary glands and afterwards be returned back into the feeding site and host in the way similar to immunoglobulin-binding proteins (IGBPs). These proteins help the tick to bind and excrete IgG ingested in blood back into the host’s body. Part of IgG (and other plasma proteins) gets from the

gut into the hemolymph and thus can bind to internal organs. In females of *Rhipicephalus appendiculatus*, IGBPs occur in hemolymph and salivary glands and via saliva they get IgG back into the host. It is proposed that IGBPs from hemolymph recognize IgG and those from salivary glands participate in excretion. Besides *R. appendiculatus*, IGBPs were isolated also from *Amblyomma variegatum*, *I. hexagonus*, *I. scapularis*, and *I. ricinus* (Wang and Nuttall 1999). Nevertheless, this function is less probable in D-Al protein due to the size of the protein with bound antibodies and its crossing from the gut into the hemolymph and further transferring to salivary glands. Rather than this, IgE is recognized and "neutralized" by D-Al protein directly in the gut, or in hemolymph where a small portion of IgE could get to. Or, the reason for D-Al protein recognition of antibodies could be its similarity to a motif or a molecule specifically recognized by IgE.

Thus, D-Al protein was predicted to posses the lipid binding site – possibly for a hydrophobic bacterial molecule, as well as IgE binding site, that was also described in other mite allergens. The lipid binding site is predicted to be located between the two β-sheets based in its homology to Der f 2 allergen (Fig. 12).

However, are there any allergens or allergenic molecules already described in ticks? Observations of allergic reactions in people bitten by tick are not so often but sometimes even anaphylactic shocks development is documented. Allergic reactions to bites of soft tick *A. reflexus* (Rolla et al. 2004), and hard ticks *I. holocyclus* (Gauci et al. 1989), *I. pacificus* (Van Wye et al. 1991), *I. ricinus* (Beaudouin et al. 1997; Fernández-Soto et al. 2001), and *R. sanguineus* (Valls et al. 2007) together with specific IgE presence in the blood of tick-biten people were reported. In the majority of cases, the molecule responsible for allergy reaction development is not known with the exception of *A. reflexus* where allergen Arg r 1 was defined. This 143 aa long molecule (after signal peptide cleavage) with the molecular weight of 15.3 kDa, without N-glycosylations, forming eight β-sheets and a random coil at C-terminus, belongs to lipocalin family (Hilger et al. 2005). Lipocalins are extracellular proteins with molecular weight of about 20 kDa that differ greatly in their structures and functions. They participate in modulation of immune response, retinol transport, invertebrate cryptic coloration, olfaction, pheromone

transport, prostaglandin synthesis, regulation of cell homoeostasis, and in the general clearance of endogenous and exogenous compounds. They are also able to bind small hydrophobic molecules, interact with receptors or form macromolecular complexes (Flower 1996). Lipocalins were identified in many tick species (for example *I. scapularis*, *I. pacificus*, *O. coriaceus*, *R. sanguineus*, *I. persulcatus*). Very often their expression is induced by blood feeding and salivary glands are defined as a site of their expression (Valenzuela et al. 2002; Francischetti et al. 2005, 2008; Anatriello et al. 2010; Konnai et al. 2011). Tick lipocalins are the example of molecules modulating host immune response. The characterized tick lipocalins are represented by moubatin, inhibitor of collagen-stimulated platelet aggregation, and OmCI, inhibitor of complement system, of *O. moubata* (Waxman and Connolly 1993; Roversi et al. 2007), toxins of *O. savigniy* (Mans et al. 2003), monomine and monotonin binding histamine and serotonin, respectively, of *A. monolakensis* (Mans et al. 2008) in soft ticks; and histamine-binding proteins of *R. appendiculatus* (Paesen et al. 2000), serotonin- and histamine-binding protein of *D. reticulatus* (Sangamnatdej et al. 2002), and lipocalin of *I. ricinus* binding leukotrienes B4 (Beaufays et al. 2008) in hard ticks. Almost all known tick lipocalins were found in salivary glands, but savicalin of *O. savigniy* is the first lipocalin found in hemocytes (Cheng et al. 2010). Although lipocalins and ML proteins in ticks have some similar features, similar size with β-rich fold, hydrophobic molecules binding, time of expression, the differences between them are obvious and they are in the site of expression and possible functions. The main function of tick lipocalins is modulation of host immune response, while ML proteins seem to be involved in tick immune processes.

It is possible that more ML proteins could be found in *I. ricinus* using the database search. More than 10 putative molecules containing ML domain have already been revealed during the genome analysis of *I. scapularis*. The majority of *I. scapularis* ML proteins were found in salivary glands. So, most probably, salivary glands of *I. ricinus* produce more such molecules at least. In this work, however, our attention was dedicated only to two ML domain containing proteins. A further interesting aim is the determination of tick ML proteins tertiary structure. Predicted models showed β-rich structure with two sheets and a putative lipid binding cavity between them. A possible hydrophobic cavity

indicates the existence of ligand(s) that differ(s) from those known in the most homologous proteins, especially in the case of IrML. The *E. coli* binding by D-Al protein showed that the molecule has a similar feature like Der f 2 allergen. As the Der f 2 ability to bind LPS was revealed (Ichikawa et al. 2009), it is possible that D-Al protein also binds just this bacterial molecule. Of course, further question is what happens with LPS (or another molecule) next. Is it "presented" for processing by immune mechanisms, acts as opsonin, or is it transported and stored at some safe place? If it is involved in immunity processes, it could start a pahway leading to production of compounds triggering the response against Gram-negative bacteria. Also, the changes in the site of expression (type of cells) or the transfer of the proteins to another tissues or organs could help in revelation of the function of *I. ricinus* ML proteins. Differences in the place of molecules occurrence can indicate their ablility to act as transport molecules and to work in more tissues. However, the two main possible functions of *I. ricinus* ML domain containing proteins, such as immune-related and/or transfer molecules are still open for discussion. What is the real function of these molecules could be the objective of future work.

5. Conclusions

ML-domain containing protein (IrML)

- IrML belongs into the group II of ML protein family. The closest to IrML related protein member of the family with known crystal structure is Npc2 with the homology of 25%.
- The gene expression is induced solely by blood feeding of the tick in all developmental stages.
- The major site of gene expression in *I. ricinus* is the gut but it can be found in hemolymph and salivary glands as well in lower level. Expression in the gut starts right after feeding begins, while the salivary glands-expression has a delay of some days.
- IrML is produced by secretory acini of salivary glands of partially fed females, by acinus type II and III.
- The *IrML* expression in tick cell line IRE/CTMV 19 is significantly influenced by the presence of Gram-negative bacteria, namely *E. coli* and *B. burgdorferi*. Impact of Gram-positive bacterium *M. luteus* and fungus *C. albicans* on the gene expression was not shown.
- The antimicrobial activity of recombinant IrML on both Gram-positive and Gram-negative bacteria was not observed under conditions used.
- On a basis of experimental results the possible function of IrML in *I. ricinus* was defined as pathogen recognition molecule.

Der-p2 allergen-like protein (D-Al protein)

- D-Al protein of *I. ricinus* is a member of group II of ML protein family. The closest to D-Al related member of this family with defined crystal structure is Der f 2 allergen of house dust mite *D. farinae* (homology (33%).
- Expression of gene encoding D-Al protein in *I. ricinus* is activated rapidly by tick blood intake in all developmental stages.
- The expression is strongly gut-specific; weak expression is observed in hemolymph.

- The presence of microorganisms (*M. luteus*, *E. coli*, *B. burgdorferi*, and *C. albicans*) has no effect on expression of gene encoding D-Al protein in tick cell line IRE/CTMV 19.
- D-Al protein binds to specific bacterial molecule of *E. coli* and also, in lower degree, of *M. luteus*.
- The antimicrobial activity of recombinant D-Al protein on both Gram-positive and Gram-negative bacteria was not observed.
- Recombinant D-Al protein interacts with immunoglobulin E in a dose-dependent manner.
- On a basis of experimental results the possible function of D-Al protein in *I. ricinus* was defined as transport molecule.

6. Materials and methods

6.1 Ticks

I. ricinus ticks were provided by the Biological Centre, Institute of Parasitology, Academy of Sciences of the Czech Republic. Uninfected ticks were reared for several generations in the animal facilities of the Institute and fed on guinea pigs that were raised to be free of infection under the strict hygiene regulations of the Central Commission for the Protection of Animals (§21, section 3e, law 246/1992). Blood-fed larvae, nymphs, and females were obtained by attaching a container (cell) with ticks to a shaved area on the back of an adult uninfected guinea pig and left on the animal until the specified engorgement stage. After the separation of the fed ticks from the guinea pig, RNA was isolated from them using TRI Reagent (Sigma, USA). The fully engorged females were kept at 4°C for another three days for blood digestion prior to RNA isolation.

6.2 First strand cDNA synthesis and polymerase chain reaction (PCR)

First strand cDNA was synthesized using RevertAid™ H Minus First Strand cDNA Synthesis Kit (MBI Fermentas, Lithuania) according to the protocol provided by the manufacturer. Primers ML1F/ML1R and AL1F/AL1R were designed according to the known partial nucleotide sequences of the gene encoding *I. ricinus* ML-domain containing protein (AY323234) and of gene encoding *I. ricinus* allergen-like protein (AJ547805) used for amplification (ML1F 5′-ATGGCCGGTTCTATGGTATTC-3′/ML1R 5-AAATTCTCCGGC TTCAGTTGTC-3′, AL1F 5′-ATGTTCCGTTACGTTGTGTTCC-3′/AL1R 5-CATCCCCACCGCTGTAAC-3′, Generi Biotech, Czech Republic).

6.3 Cloning and sequencing

Amplified PCR products were cloned into a pCR4-TOPO vector (Invitrogen, USA) directly or after purification from the gel by QIAquick Gel Extraction Kit (Qiagen, USA) according to the manufacturer's protocol. Competent *E. coli* cells (Invitrogen, USA) were transformed by recombinant constructs, plated on LB/agar/Amp and incubated overnight at 37°C. Individual recombinants were randomly picked from the plates, grown in small volume

(3 ml) of LB/amp and the plasmid DNA was purified using the Plasmid Miniprep Kit (Qiagen, USA). Inserts were sequenced in both directions using M13 primers set. Sequencing was performed using ABI 3130 Sequencer and the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

6.4 RT-PCR analysis of stage specific and tissue specific patterns and time expression of the genes

RT-PCR was performed using the Enhanced Avian HS RT-PCR kit (Sigma, USA). The equal amount of total RNA isolated from unfed and engorged larvae, nymphs and females of *I. ricinus* and different tissues (salivary glands, gut, ovaries, Malpighian tubules, and hemolymph) of partially engorged females (five days) was used as a template in RT-PCR in order to reveal the *IrML* and *D-Al* gene expression in different developmental and feeding stages. To determine time expression of the gene, equal amounts of total RNA from unfed, 1, 3, 5, 7 days-fed, and totally engorged adult females, were used as templates in RT-PCR. Also, an equal amount of RNA from salivary glands and gut from females unfed, 1, 2, 4 and 6 days after feeding, were used as templates in other PCRs (for *IrML*). The primers, ML2F 5′-CACCATGGCCGGTTCTATGGTA-3′/ML2R 5′- CTATTGCTTCAGCTCTA CCG-3′ for *IrML*, and AL2F 5′-CACCATGTTCCGTTACGTTGTGTTCC-3′/AL2R 5′-TCACTCGATGACGATGTCCGA-3′ for *D-Al* were used for the amplification with an annealing temperature of 55° C. A constitutively-expressed actin gene (AY333957) was chosen as a control. The control amplification was performed at the annealing temperature of 55° C with the primers Act-F1 5'-CGTCTGGATCGGCGGCTCTAT-3' and Act-R1 5'-ACGCGCACTCTTTTCCACAATCTC-3'. All RNAs used were first checked for DNA contamination by adding them directly into the PCR reaction with the specific primers.

6.5 Bioinformatics/sequence analysis

Sequences were compared against the GenBank non-redundant (NR) protein database using the BlastX program from the executable package at ftp://ftp.ncbi.nlm.nih.gov/blast/executables (Altschul et al. 1997) and searched

against the Conserved Domains Database (CDD) (ftp://ftp.ncbi.nlm.nih.gov/pub/mmdb/cdd), which includes all Pfam (Bateman et al. 2000) and Smart (Schultz et al. 1998) protein domains, as well as against the VectorBase (Lawson et al. 2009) (http://www.vectorbase.org) and the Gene Index Project (Quackenbush et al. 2001) (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi). Protein secondary structure prediction was performed by the PSIpred program (http://bioinf.cs.ucl.ac.uk/psipred) (McGuffin et al. 2000). Putative signal peptide, possible sites of O-glycosylation, N-glycosylation, and phosphorylation were determined with the prediction servers Signal P 3.0 (http://www.cbs.dtu.dk/services/SignalP) (Bendtsen et al. 2004), NetOGlyc 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc) (Julenius *et al.* 2005), NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc) and NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos) (Blom *et al.* 1999), respectively.

6.6 Tertiary structure prediction

For prediction of the 3D structure of the IrML protein, CPHmodel 2.0 Server was used (http://www.cbs.dtu.dk/services/CPHmodels) to find the homologues for the *I. ricinus* molecule. A pdb file of the molecule (according to the similar sequences of structures from the protein databank) was downloaded and used in Polyview-3D for protein visualization (http://polyview.cchmc.org/polyview3d.html) (Porollo and Meller 2007).

6.7 Expression and purification of the recombinant proteins

The gene encoding Der-p2 allergen-like protein without signal peptide, prepared by PCR using the primers AL3F 5′-CACCAGCGCAGAAAAGCCGT-3′ and AL2R (sequence above), and the gene of ML-domain containing protein without signal peptide, obtained from PCR with primers ML3F 5′-CACCCGCTGACGCACTTTCA-3′ and ML2R (sequence above), were expressed in pET100/D-TOPO (Invitrogen, USA) expression vector as recombinant fusion proteins with N-terminal 6xHis tag. Rosetta-gami competent cells (Novagen, Germany) were transformed by recombinant construct. All procedures were performed according to the manufacturer's protocol in Champion pET directional TOPO expression kits (Invitrogen, USA). Isopropyl-β-d-thiogalactopyranoside (IPTG) (1mM final concentration)

was used for the induction of recombinant protein expression. Recombinant Der-p2 allergen-like protein was purified by Co^{2+}-affinity chromatography using cartridge Co-MAC (Novagen, Germany). Bacterial lysate was prepared using lysis buffer containing 8 M urea, 0.1 M NaH_2PO_4, 0.01M Tris-HCl, pH 8.0. The purification under denaturing conditions was performed according to the manufacturer's protocol: cartridge was equilibrated with 5 volumes of binding buffer (6 M urea, 0.5 M NaCl, 0.02 M Tris-HCl, 0.005 M imidazole, pH 7.9), and then the sample in binding buffer was applied. The cartridge was washed with 10 volumes of binding buffer, 6 volumes of washing buffer (6 M urea, 0.5 M NaCl, 0.02 M Tris-HCl, 0.06 M imidazole, pH 7.9) and the recombinant protein was eluted with 6 volumes of elution buffer (6 M urea, 0.5 M NaCl, 0.02 M Tris-HCl, 1 M imidazole, pH 7.9). The elution fractions were checked on SDS-PAGE and fractions containing recombinant protein given for further tests were dialyzed against PBS (phosphate-buffered saline) buffer, pH 7.4 with 6 M urea, 4 M urea, 2 M urea, 1 M urea, 0.5 M urea and PBS, respectively. The presence of the His-tagged Der-p2 allergen-like protein was verified by immunodetection with Ni-NTA conjugates (Qiagen, Germany).

6.8 SDS-PAGE and immunodetection of recombinant protein

SDS-PAGE under reducing conditions was performed as described by Laemmli (1970). Separated proteins were stained by PageBlue™ Protein Staining Solution (MBI Fermentas, Lithuania). Proteins were transferred onto Immobilon-P transfer membrane (Millipore, USA). The blots were stained with Ni-NTA conjugates (Qiagen, USA) that detects 6xHis tag of recombinant protein, according to manufacturer's protocol.

6.9 Immunization of mouse and serum preparation

Recombinant protein in PBS (100 µl) was mixed with the same volume of Freund's incoplate adjuvans and used for immunization of adult mouse Balb/c in 10-day intervals for four times. Serum was prepared from blood incubated in room temperature for 3 hours and centrifuged (2500 rpm, 20 min, 4°C). Supernatant was removed and used in experiments.

6.10 Immunodetection

Membrane with immobilized proteins was washed two times for 10 min in PBS, blocked 1 hour in 3% non-fat milk in PBST (PBS with 0.05% Tween-20), washed three times for 10 min in PBST and incubated for 2 hours with mouse serum (diluted from 1:20 to 1:1000 in PBST). Then, it was washed again in PBST (three times for 10 min), incubated for 1 hour with secondary antibody GAM/Px (Sigma) diluted 1:1000 in PBST and washed by PBST (once for 10 min, twice for 5 min). Hydrogen peroxide was used as a substrate and 4-chloro-1-naftol as a chromogen.

6.11 Immunoprecipitation

One ml of homogenized ticks was combined with 50 µl of antibody and the reaction was incubated overnight at 4°C. One hundred µl of immobilized protein A/G resin slurry (Pierce, USA) was added to a microcentrifuge tube and briefly centrifuged to pellet resin; the supernatant was discarded. For two times repetition, 0.5 ml of IP buffer (25mM Tris, 150mM NaCl, pH 7.2) was added, briefly centrifuged and supernatant discarded. The antigen-antibody complex was added to the resin and incubated with gentle mixing for 2.5 hours at room temperature. 0.5 ml of IP buffer was added, briefly centrifuged and supernatant discarded. This step was repeated two times. To elute the immune complex, 50 µl of elution buffer (0.15M glycine•HCl buffer, pH 2.7) was added and incubated for 5 minutes, tube was briefly centrifuged and the supernatant collected. This step was repeated and the two supernatant fractions were combined. Eluate was adjusted to physiological pH by adding 10 µl of the neutralization buffer (1M Tris, pH 9) per 100 µl of eluate. The immunoprecipitation products were used directly for SDS-PAGE.

6.12 Antimicrobial activity determination

Double-layer technique was used to estimate antimicrobial activity of the recombinant proteins (Čeřovský *et al.* 2008). The plates were incubated at 37°C for 6 hours with repeated checking for the growth of bacteria (*Escherichia coli*, *Micrococcus luteus*).

6.13 *In vitro* binding assay I – bacteria and recombinant proteins

Protocol of Ichikawa et al. (1998) was used in the experiment. Bacteria (*E. coli*, *M. luteus)* were grown to late log phase, collected by centrifugation (2,500g, 10 min), and suspended in 1/10 of the original volume with 0.9% NaCl. An equal volume of 20% acetic acid was added, and the bacteria were incubated at room temperature for 5 min. Five volumes of 1 M Tris-HCl (pH 8.2) were added, and the bacteria were spined (2,500g, 10 min) and resuspended in one-tenth of the original culture volume in 10 mM Tris-HCl (pH 8.2). An equal volume of recombinant D-Al protein in concentration of 100 µg/ml was added to the bacterial suspension and incubated at room temperature for 3 or 30 min. The suspension was centrifuged for 2 min at 10,000g, the pellet was washed twice in water and then suspended in PBS and sample buffer for electrophoresis. Sample was denatured and run on SDS-PAGE.

6.14 *In vitro* binding assay II – bacteria and tick cell line

Selected microorganisms (*M. luteus*, *E. coli*, *C. albicans*, *B. burgdorferi*) were inactivated at 100°C for 5 min and cooled down. They, in the concetration of 10^8 cells/ml, were added to tick cell line IRE/CTMV 19 in the concentration of $5*10^6$ cells/ml and incubated for 18 hours. Then, RNA of the cell mixture was isolated and RT-PCR performed to detect expression of studied genes.

6.15 IgE binding reactivity

Purified recombinant Der-p2 allergen-like protein was diluted to 10 µg/ml in PBS. Each well of a 96-well microplate (Maxisorp F96 MicroWell Plate, Nunc, Denmark) was coated with 50 µl of the recombinant protein and incubated at 4°C overnight. All further incubations were performed at room temperature with at least five washes with PBS containing 0.05% Tween 20 (PBST) between them. Fifty microlitres of human biotinylated IgE (BioPorto Diagnostic, Denmark) in different concentrations (0.1, 1, 3, 5, 10 µg/ml) were added into a microplate and incubated for 1 hour. Then, 50 µl of streptavidin-peroxidase (Sigma, USA) diluted 1:1000 in distilled water was added to each well and incubated for 30 min. Orthophenylenediamine (Sigma, USA) with hydrogen peroxide (50 µl/well) were used as a substrate for the enzymatic

colour reaction. The reaction was performed in the dark for 15 minutes and stopped by the addition of 50 µl of 1 M H_2SO_4. Optical densities were measured at 490 nm with an ELISA spectrophotometer (Multiskan MCC 340, Labsystems Oy, Finland). Bovine serum albumin (BSA) and a recombinant protein produced in prokaryotic expression system were used as controls.

6.16 Statistical analysis

The significance of any differences obtained between single protein groups and used different antibody concentrations was evaluated by the factorial ANOVA with a subsequent Tukey HSD test (Statistica 6.0, Statsoft, Inc. Tulsa, USA). The presented results represent three similar experiments.

6.17 Preparation of *in situ* hybridization (ISH) probes

A specific antisense DNA probe was prepared using the PCR digoxigenin (DIG) Probe Synthesis Kit (Roche Applied Science, Germany). The cDNA amplified using ML2F/ML2R and AL2F/AL2R primers served as template for probe synthesis. The DIG labeled probe was produced by asymmetric PCR from 300 ng of the obtained cDNA and gene-specific reverse primer (ML2R or AL2R) in a final reaction volume of 25 µl. The initial denaturation step (95° C for 2 min) was followed by ten cycles of 95° C for 30 s, 52° C for 30 s, and 72° C for 1.5 min, and subsequently by 11 cycles of 94° C for 30 s, 52° C for 30 s, and 72° C for 2.5 min. The DIG-labeled probe was gel purified and stored at -20° C. The specific sense probe was synthesized using the same protocol but with the ML2F or AL2F primer.

6.18 *In situ* hybridization (ISH)

Dissected tick organs were fixed in 4% paraformaldehyde at 4° C overnight and subjected to ISH as previously described by (Kim et al. 2006). The color reaction was observed under a binocular microscope and stopped by repeated washes in PBS (phosphate buffered saline) with 0.2% Tween 20, pH 7.2 (PBST) followed by a wash in PBS:glycerol (1:1) for 20 min.

Stained tissues were mounted in 100% glycerol on glass slides and observed under a fluorescence microscope Olympus BX51 with Nomarski DIC optics and attached Olympus DP70 digital camera (Olympus, Japan). Tissues

from five to ten ticks were studied for each feeding stage and used for hybridization. Two negative controls were performed. The hybridization step was omitted in one of the controls, and the specific sense probe instead of the antisense probe was used in the protocol. Images were processed using with the program Paint.NET v3.5.1.

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8. Appendix

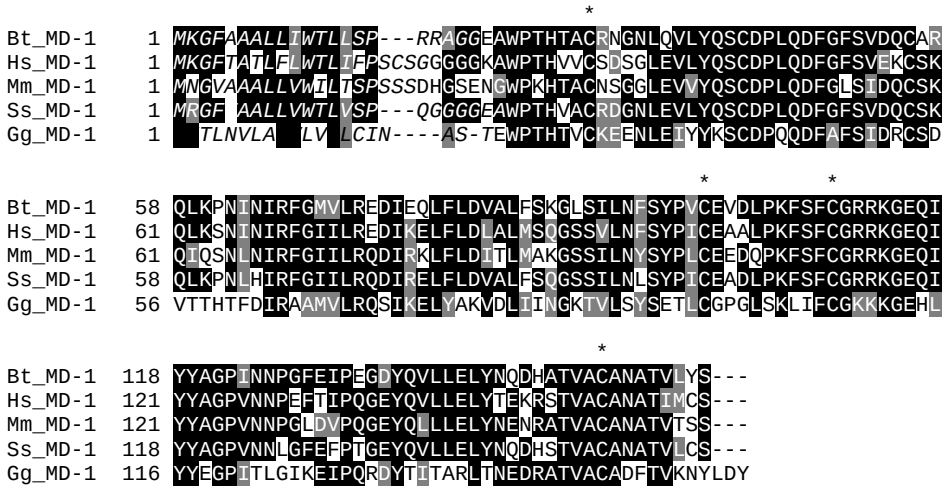


Fig. 15. Alignment of group I members of ML protein family – MD-1 protein orthologs. Signal peptides are written in italics and conserved cysteine residues are marked by asterisks. *Bos taurus*, NP_001077230 (Bt_MD-1); *Homo sapiens*, AAC98152 (Hs_MD-1); *Mus musculus*, BAA32399 (Mm_MD-1); *Sus scrofa*, BAF48332 (Ss_MD-1); *Gallus gallus*, Q90890 (Gg_MD-1).

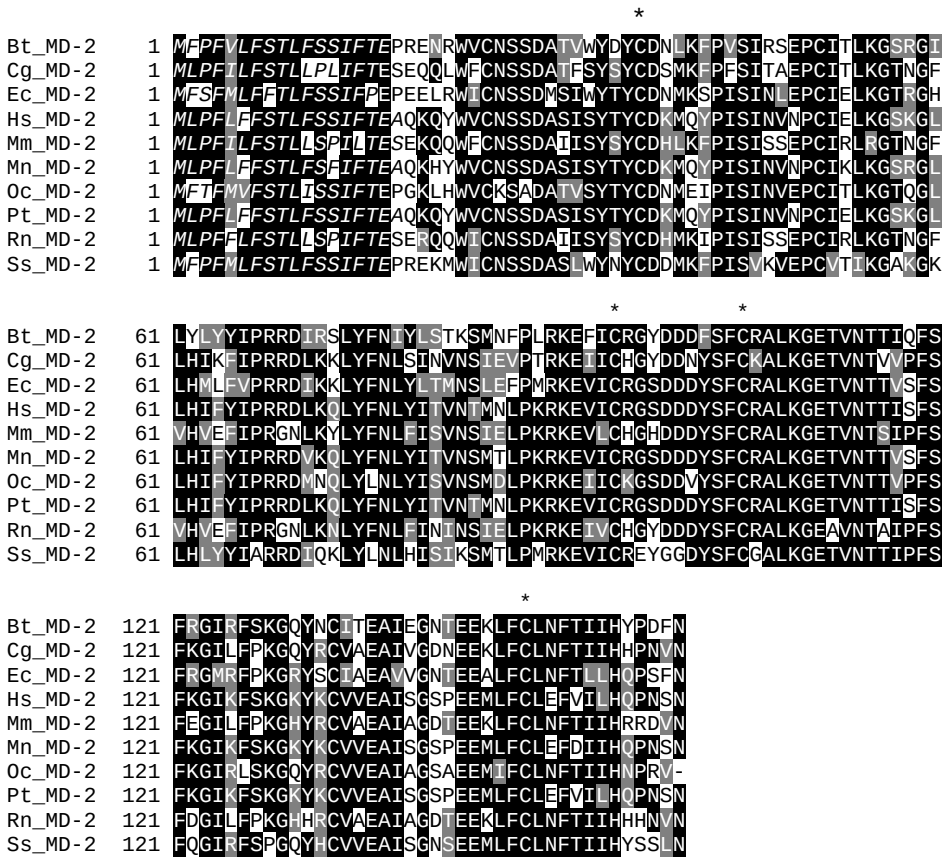


Fig. 16. Alignment of group I members of ML protein – MD-2 protein orthologs. Signal peptides are written in italics and conserved cysteine residues are marked by asterisks. *Bos taurus*, BAC67682 (Bt_MD-2); *Cricetulus griseus*, AAK57984 (Cg_MD-2); *Equus caballus*, AAQ95744 (Ec_MD-2); *Homo sapiens*, BAA78717 (Hs_MD-2); *Mus musculus*, BAA93619 (Mm_MD-2); *Macaca nemestrina*, ADF81051 (Mn_MD-2); *Oryctolagus cuniculus*, AAM50061 (Oc_MD-2); *Pan troglodytes*, NP_001123946 (Pt_MD-2); *Rattus norvegicus*, NP_001019450 (Rn_MD-2); *Sus scrofa*, BAG12311 (Ss_MD-2).

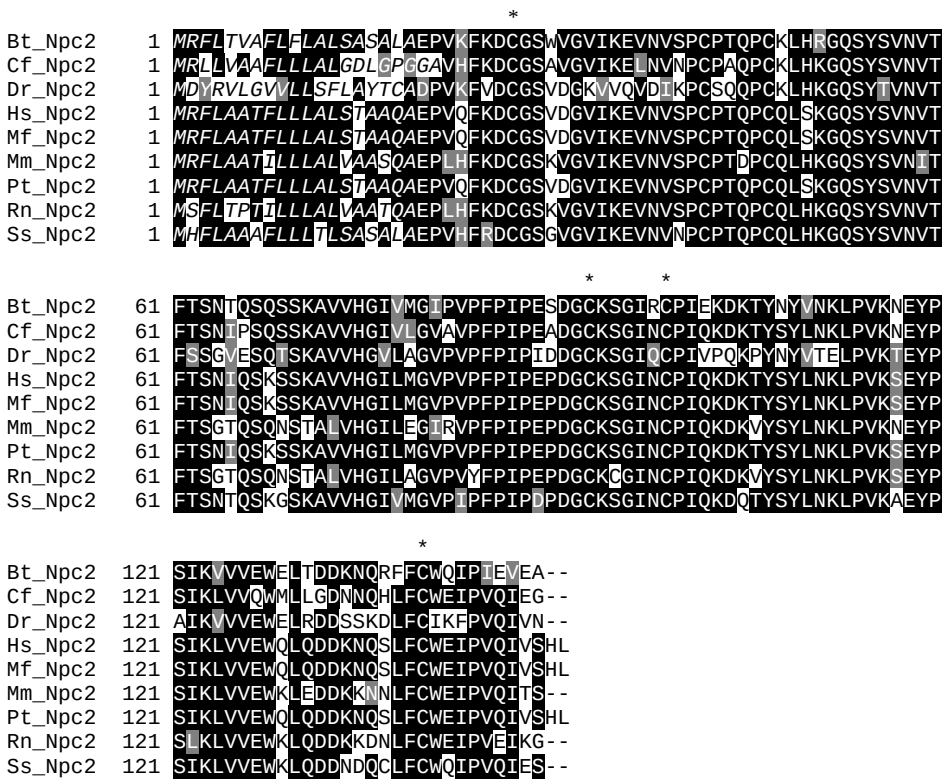


Fig. 17. Alignment of group II members of ML protein family – Niemann-Pick type C2 proteins. Signal peptides are written in italics and conserved cysteine residues are marked by asterisks. *Bos taurus*, P79345 (Bt_Npc2); *Canis familiaris*, Q28895 (Cf_Npc2); *Danio rerio*, Q9DGJ3 (Dr_Npc2); *Homo sapiens*, P61916 (Hs_Npc2); *Macaca fascicularis*, P61918 (Mf_Npc2); *Mus musculus*, AAH07190 (Mm_Npc2); *Pan troglodytes*, P61917 (Pt_Npc); *Rattus norvegicus*, EDL81526 (Rn_Npc2); *Sus scrofa*, O97763 (Ss_Npc2).

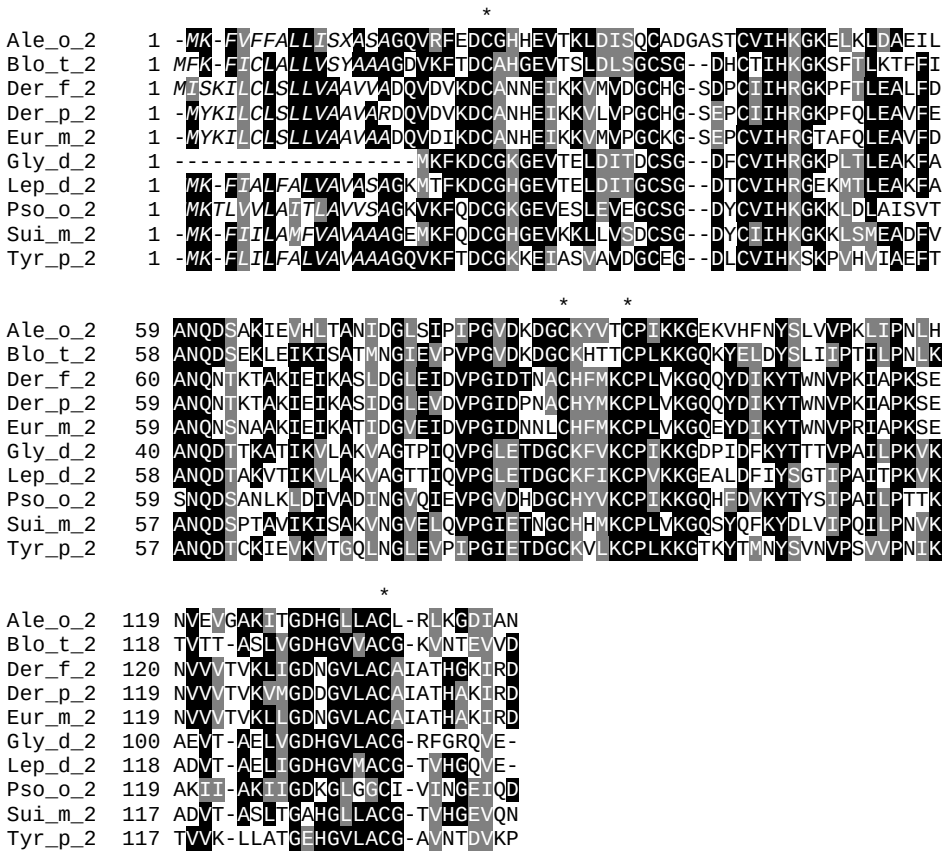


Fig. 18. Alignment of group II members of ML protein family – allergens of storage and house dust mites. Signal peptides are written in italics and conserved cysteine residues are marked by asterisks. *Aleuroglyphus ovatus*, AAS75832 (Ale_o_2); *Blomia tropicalis*, ABG76185 (Blo_t_2); *Dermatophagoides farinae*, BAA01240 (Der_f_2); *D. pteronyssinus*, AAF86462 (Der_p_2); *Euroglyphus maynei*, AAC82349 (Eur_m_2); *Glycyphagus domesticus*, CAB76459 (Gly_d_2); *Lepidoglyphus destructor*, CAA61419 (Lep_d_2); *Psoroptes ovis*, Q965E2 (Pso_o_2); *Suidasia medanensis*, AAS75831 (Sui_m_2); *Tyrophagus putrescentiae*, O02380 (Tyr_p_2).

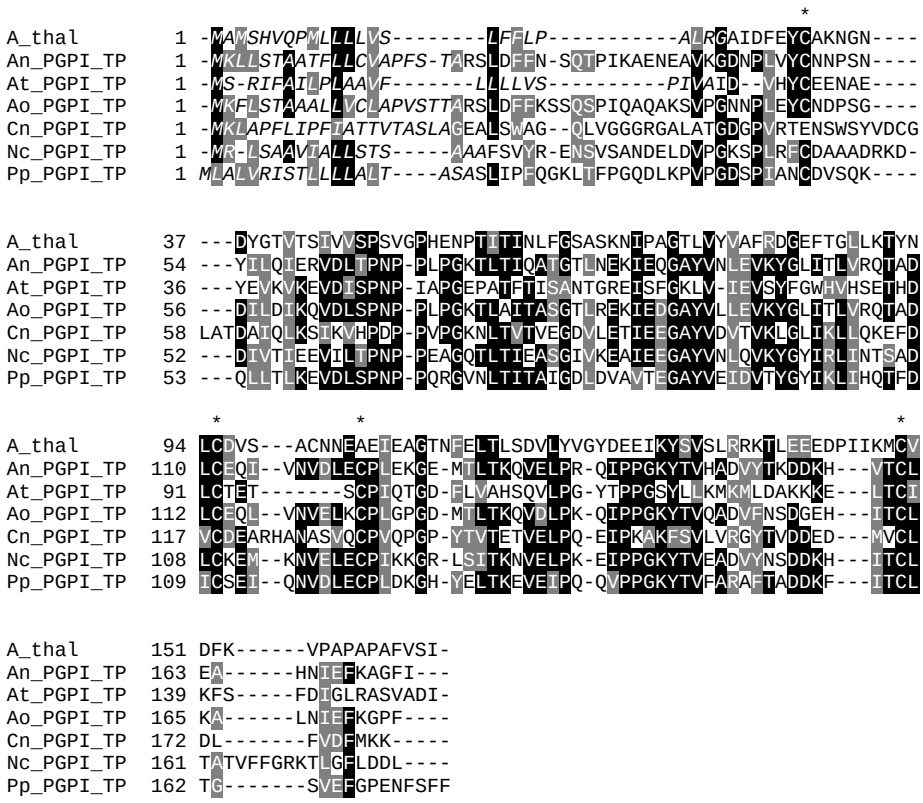


Fig. 19. Alignment of group III members of ML protein family – *Arabidopsis thaliana* ML protein and phosphatidylglycerol/phosphatidylinositol transfer proteins. Signal peptides are written in italics and conserved cysteine residues are marked by asterisks. *A. thaliana*, AY050390 (A_thal); *Aspergillus niger*, XP_001399467 (An_PGPI_TP); *A. oryzae*, AAD16095 (Ao_PGPI_TP); *A. thaliana*, AED91022 (At_PGPI_TP); *Cryptococcus neoformans*, P0CP28 (Cn_PGPI_TP); *Neurospora crassa*, Q7RZ85 (Nc_PGPI_TP); *Pichia pastoris*, CAY68596 (Pp_PGPI_TP).

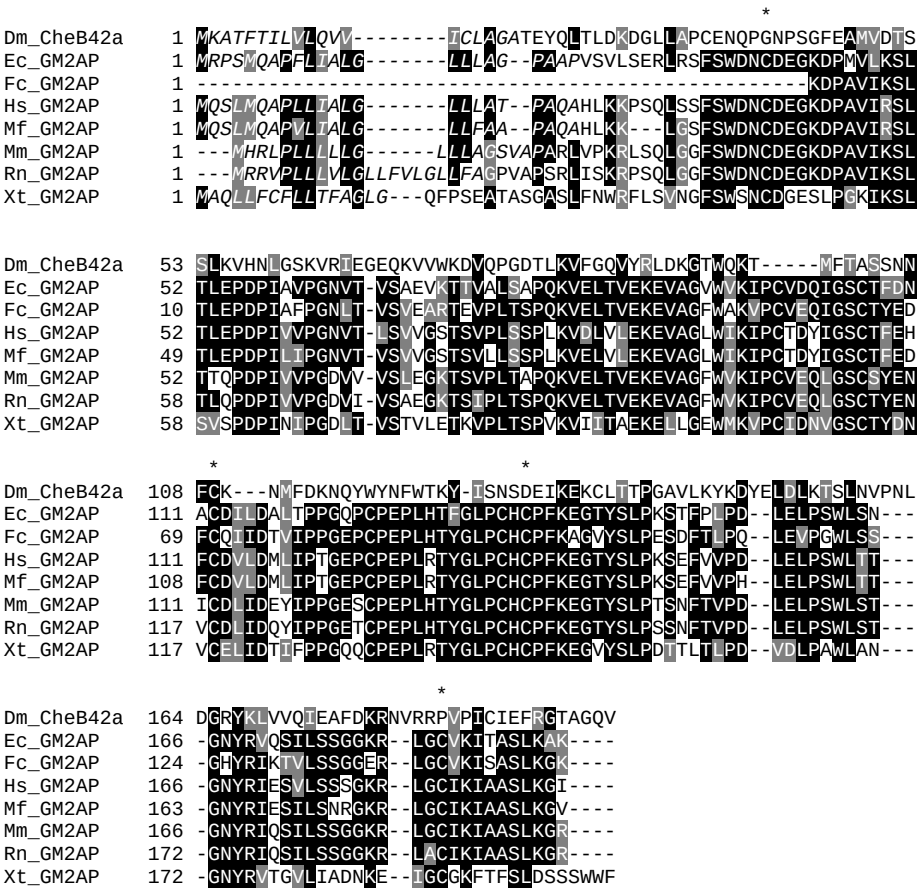
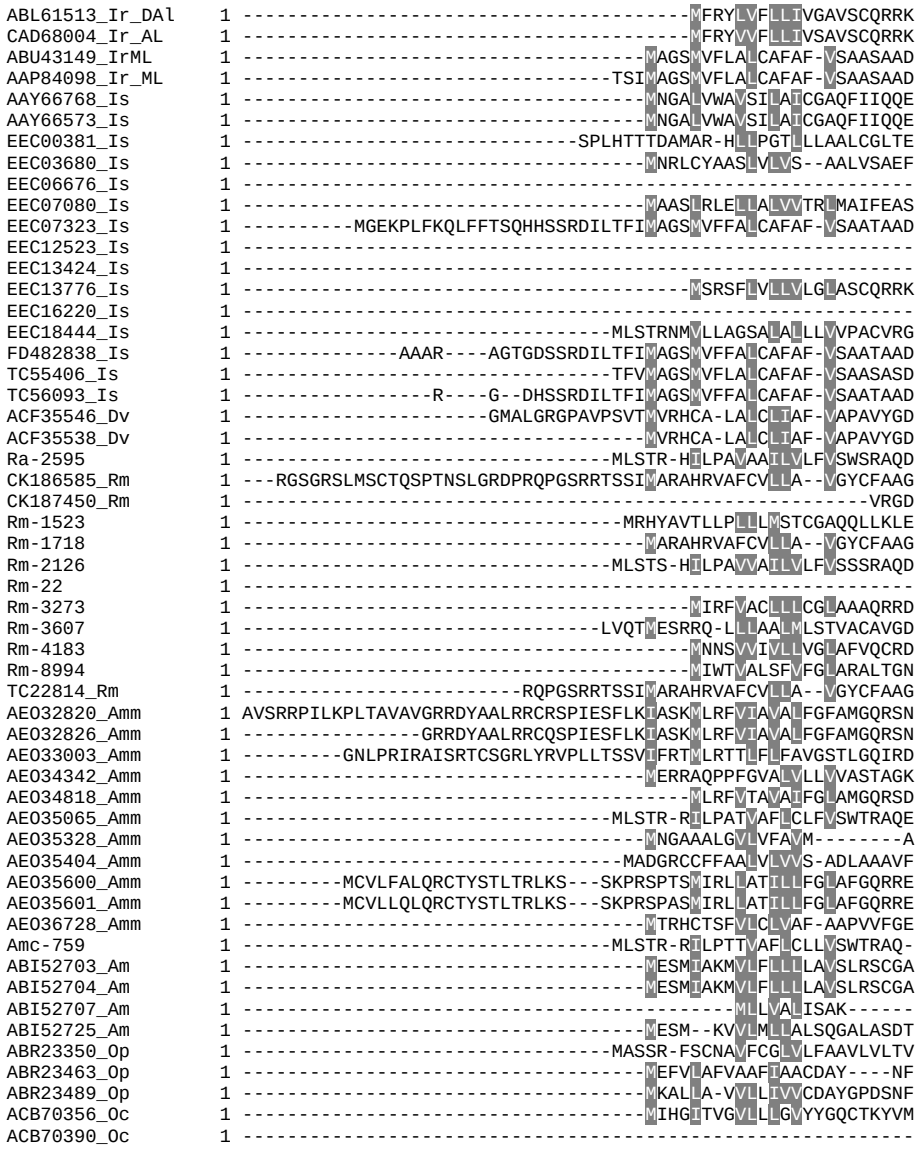


Fig. 20. Alignment of group IV members of ML protein family – perception protein of *Drosophila* and GM2 activator proteins. Signal peptides are marked in green and conserved cysteine residues by asterisks. *D. melanogaster*, NP_995765 (Dm_CheB42a); *Equus caballus*, NP_001075381 (Ec_GM2AP); *Felis cattus*, AAS64350 (Fc_GM2AP); *Homo sapiens*, CAA43993 (Hs_GM2AP); *Macaca fascicularis*, BAD51960 (Mf_GM2AP); *Mus musculus*, AAA21543 (Mm_GM2AP); *Rattus norvegicus*, BAC24018 (Rn_GM2AP); *Xenoplus tropicalis*, CAJ83929 (Xt_GM2AP).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EEC16220_Is 1 ----------
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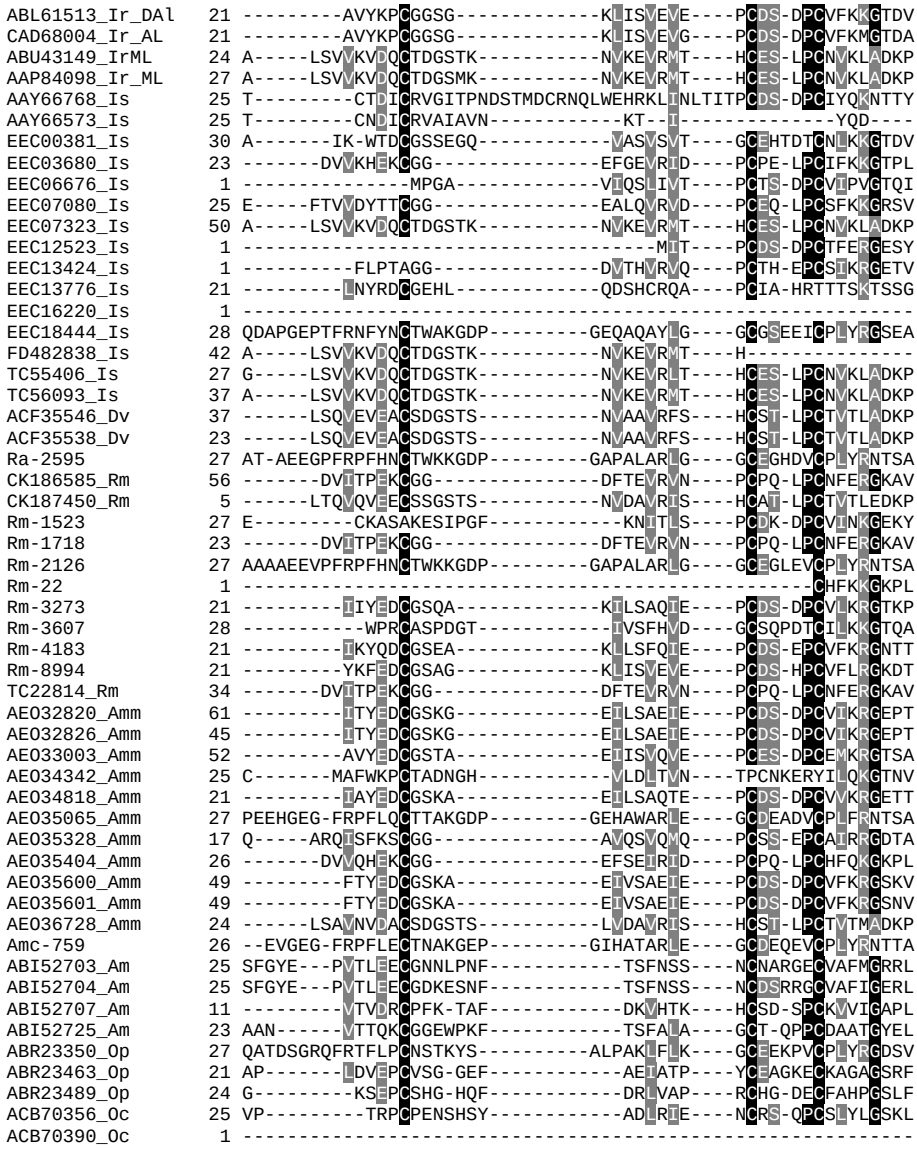
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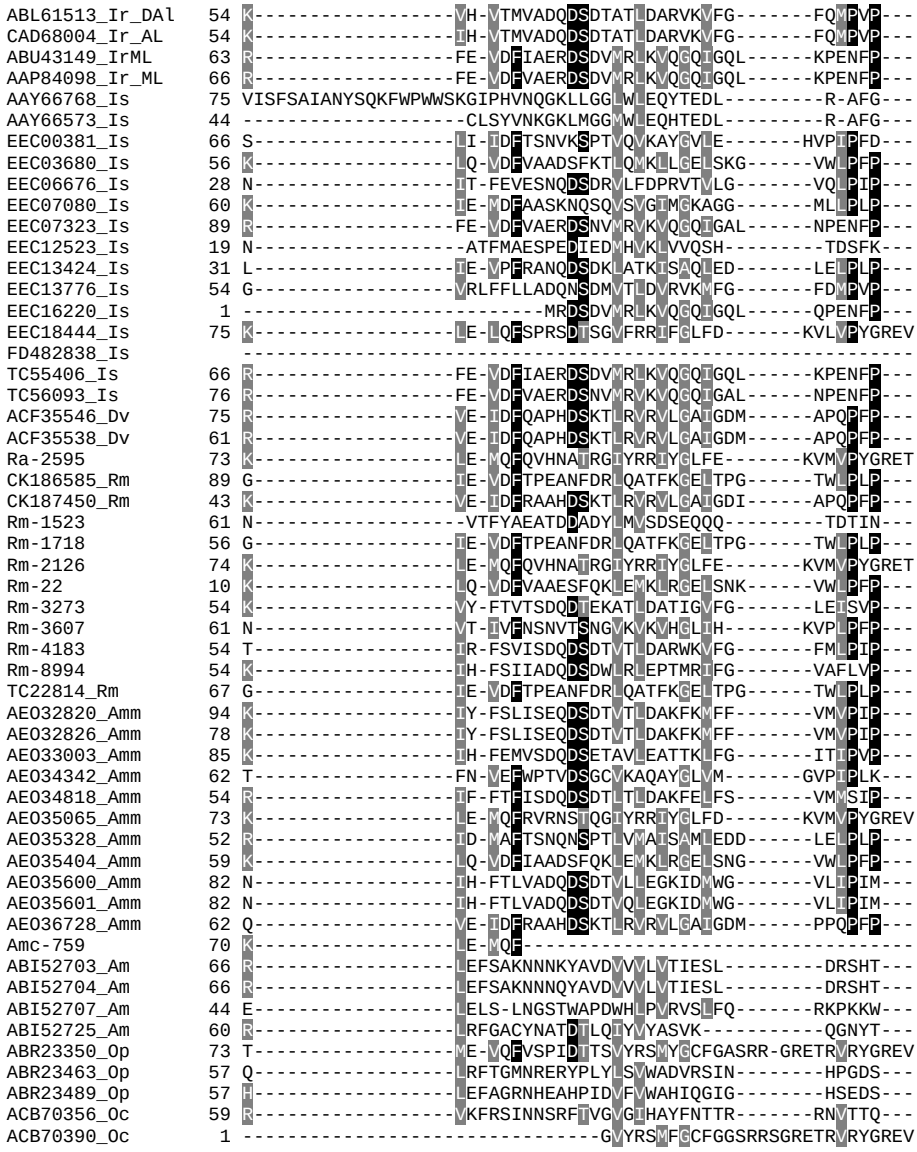
EEC13424_Is 1 ----------FLPTAGG---------------DVTHVRVQ----PCTH-EPCSIKRGETV
EEC13776_Is 21 ---------LNYRDGGEHL-------------QDSHCRQA----PCIA-HRTTTSKTSSG
EEC16220_Is 1 --
EEC18444_Is 28 QDAPGEPTFRNFYNGTWAKGDP---------GEQAQAYLG----GCGSEEICPLYRGSEA
FD482838_Is 42 A-----LSVVKVDQGTDGSTK-----------NVKEVRVT----H----------------
TC55406_Is 27 G-----LSVVKVDQGTDGSTK-----------NVKEVRLT----HCES-LPCNVKLADKP
TC56093_Is 37 A-----LSVVKVDQGTDGSTK-----------NVKEVRVT----HCES-LPCNVKLADKP
ACF35538_Dv 37 ------LSQVEVEAGSDGSTS-----------NVAAVRFS----HCST-LPCTVTLADKP
ACF35538_Dv 23 ------LSQVEVEAGSDGSTS-----------NVAAVRFS----HCST-LPCTVTLADKP
Ra-2595 27 AT-AEEGPFRPFHNGTWKKGDP---------GAPALARLG----GCEGHDVCPLYRNTSA
CK186585_Rm 56 -------DVITPEKGGG---------------DFTEVRVN----PCPQ-LPCNFERGKAV
CK187450_Rm 5 ------LTQVQVEEGSSGSTS-----------NVDAVRIS----HCAT-LPCTVTLEDKP
Rm-1523 27 E---------CKASAKESIPGF-----------KNITLS----PCDK-DPCVINKGEKY
Rm-1718 23 -------DVITPEKGGG---------------DFTEVRVN----PCPQ-LPCNFERGKAV
Rm-2126 27 AAAAEEVPFRPFHNGTWKKGDP---------GAPALARLG----GCEGLEVCPLYRNTSA
Rm-22 1 --CHFKKGKPL
Rm-3273 21 ---------IIYEDGGSQA-------------KILSAQIE----PCDS-DPCVLKRGTKP
Rm-3607 28 -----------WPRGASPDGT-----------IVSFHVD----GGSQPDTCILKKGTQA
Rm-4183 21 ---------IKYQDGGSEA-------------KLLSFQIE----PCDS-EPCVFKRGNTT
Rm-8994 21 ---------YKFEDGGSAG-------------KLISVEVE----PCDS-HPCVFLRGKDT
TC22814_Rm 34 -------DVITPEKGGG---------------DFTEVRVN----PCPQ-LPCNFERGKAV
AEO32820_Amm 61 ---------ITYEDGGSKG-------------EILSAEIE----PCDS-DPCVIKRGEPT
AEO32826_Amm 45 ---------ITYEDGGSKG-------------EILSAEIE----PCDS-DPCVIKRGEPT
AEO33003_Amm 52 ---------AVYEDGGSTA-------------EIISVQVE----PCES-DPCEVKRGTSA
AEO34342_Amm 25 C-------MAFWKPGTADNGH------------VLDLTVN----TPCNKERYILQKGTNV
AEO34818_Amm 21 ---------IAYEDGGSKA-------------EILSAQTE----PCDS-DPCVVKRGETT
AEO35065_Amm 27 PEEHGEG-FRPFLQGTTAKGDP---------GEHAWARLE----GCDEADVCPLFRNTSA
AEO35328_Amm 17 Q-----ARQISFKSGGG---------------AVQSVQVQ----PCSS-EPCAIRRGDTA
AEO35404_Amm 26 -------DVVQHEKGGG---------------EFSEIRID----PCPQ-LPCHFQKGKPL
AEO35600_Amm 49 ---------FTYEDGGSKA-------------EIVSAEIE----PCDS-DPCVFKRGSKV
AEO35601_Amm 49 ---------FTYEDGGSKA-------------EIVSAEIE----PCDS-DPCVFKRGSNV
AEO36728_Amm 24 ------LSAVNVDAGSDGSTS-----------LVDAVRIS----HCST-LPCTVTMADKP
Amc-759 26 --EVGEG-FRPFLEGTNAKGEP---------GIHATARLE----GCDEQEVCPLYRNTTA
ABI52703_Am 25 SFGYE---PVTLEEGGNNLPNF-----------TSFNSS----NCNARGECVAFMGRRL
ABI52704_Am 25 SFGYE---PVTLEEGGDKESNF-----------TSFNSS----NCDSRRGCVAFIGERL
ABI52707_Am 11 ---------VTVDRGPFK-TAF-----------DKVHTK----HCSD-SPCKVVIGAPL
ABI52725_Am 23 AAN------VTTQKGGGEWPKF------------TSFALA----GCT-QPPCDAATGYEL
ABR23350_Op 27 QATDSGRQFRTFLPGNSTKYS----------ALPAKLFLK----GCEEKPVCPLYRGDSV
ABR23463_Op 21 AP-------LDVEPGVSG-GEF-----------AEIATP----YCEAGKECKAGAGSRF
ABR23489_Op 24 G---------KSEPGSHG-HQF-----------DRLVAP----RCHG-DECFAHPGSLF
ACB70356_Oc 25 VP---------TRPGPENSHSY-----------ADLRIE----NCRS-QPCSLYLGSKL
ACB70390_Oc 1 --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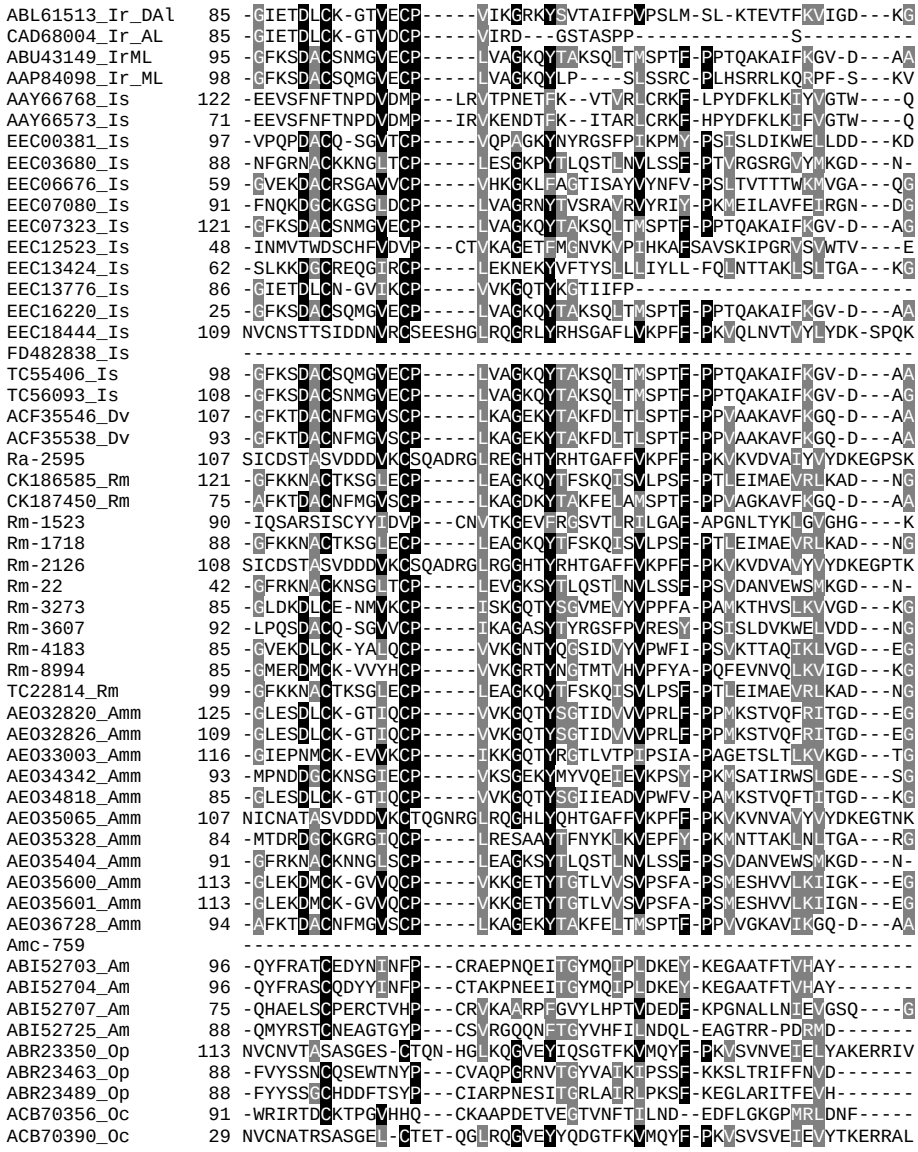
CAD68004_Ir_AL 54 K----------IH-VTMVADQDSDTATLDARVKVFG-------FQMPVP---
ABU43149_IrML 63 R----------FE-VDFIAERDSDVVRLKVQGQIGQL------KPENFP---
AAP84098_Ir_ML 66 R----------FE-VDFVAERDSDVVRLKVQGQIGQL------KPENFP---
AAY66768_Is 75 VISFSAIANYSQKFWPWWSKGIPHVNQGKLLGGLWLEQYTEDL---------R-AFG---
AAY66573_Is 44 ----------CLSYVNKGKLMGGVWLEQHTEDL---------R-AFG---
EEC00381_Is 66 S----------LI-IDFTSNVKSPTVQVKAYGVLE-------HVPIPFD---
EEC03680_Is 56 K----------LQ-VDFVAADSFKTLQMKLLGELSKG------VWLPFP---
EEC06676_Is 28 N----------IT-FEVESNQDSDRVLFDPRVTVLG-------VQLPIP---
EEC07080_Is 60 K----------IE-VDFAASKNQSQVSVGTMGKAGG-------ML-PLP---
EEC07323_Is 89 R----------FE-VDFVAERDSNVVRVKVQGQIGAL------NPENFP---
EEC12523_Is 19 N----------ATFMAESPEDIEDVHVKLVVQSH---------TDSFK---
EEC13424_Is 31 L----------IE-VPFRANQDSDKLATKISAQLED-------LELPLP---
EEC13776_Is 54 G----------VRLFFLLADQNSDMVTLDVRVKMFG-------FDMPVP---
EEC16220_Is 1 ----------MRDSDVVRLKVQGQIGQL------QPENFP---
EEC18444_Is 75 K----------LE-LQFSPRSDTSGVFRRIFGLFD-------KVLVPYGREV
FD482838_Is
TC55406_Is 66 R----------FE-VDFIAERDSDVVRLKVQGQIGQL------KPENFP---
TC56093_Is 76 R----------FE-VDFVAERDSNVVRVKVQGQIGAL------NPENFP---
ACF35546_Dv 75 R----------VE-IDFQAPHDSKTLRVRVLGAIGDM------APQPFP---
ACF35538_Dv 61 R----------VE-IDFQAPHDSKTLRVRVLGAIGDM------APQPFP---
Ra-2595 73 K----------LE-VQFQVHNATRGIYRRIYGLFE-------KVMVPYGRET
CK186585_Rm 89 G----------IE-VDFTPEANFDRLQATFKGELTPG------TWLPLP---
CK187450_Rm 43 K----------VE-IDFRAAHDSKTLRVRVLGAIGDI------APQPFP---
Rm-1523 61 N----------VTFYAEATDDADYLMVSDSEQQQ---------TDTIN---
Rm-1718 56 G----------IE-VDFTPEANFDRLQATFKGELTPG------TWLPLP---
Rm-2126 74 K----------LE-VQFQVHNATRGIYRRIYGLFE-------KVMVPYGRET
Rm-22 10 K----------LQ-VDFVAAESFQKLEMKLRGELSNK------VWLPFP---
Rm-3273 54 K----------VY-FTVTSDQDTEKATLDATIGVFG-------LEISVP---
Rm-3607 61 N----------VT-IVFNSNVTSNGVKVKVHGLIH-------KVPLPFP---
Rm-4183 54 T----------IR-FSVISDQDSDTVTLDARWKVFG-------FMLPIP---
Rm-8994 54 K----------IH-FSIIADQDSDWLRLEPTMRIFG-------VAFLVP---
TC22814_Rm 67 G----------IE-VDFTPEANFDRLQATFKGELTPG------TWLPLP---
AEO32820_Amm 94 K----------IY-FSLISEQDSDTVTLDAKFKMFF-------VMVPIP---
AEO32826_Amm 78 K----------IY-FSLISEQDSDTVTLDAKFKMFF-------VMVPIP---
AEO33003_Amm 85 K----------IH-FEMVSDQDSETAVLEATTKLFG-------ITIPVP---
AEO34342_Amm 62 T----------FN-VEFWPTVDSGCVKAQAYGLVM-------GVPIPLK---
AEO34818_Amm 54 R----------IF-FTFISDQDSDTITLDAKFELFS-------VMVSIP---
AEO35065_Amm 73 K----------LE-VQFRVRNSTQGIYRRIYGLFD-------KVMVPYGREV
AEO35328_Amm 52 R----------ID-VAFTSNQNSPTDVWAISAMLEDD------LELPLP---
AEO35404_Amm 59 K----------LQ-VDFIAADSFQKLEMKLRGELSNG------VWLPFP---
AEO35600_Amm 82 N----------IH-FTLVADQDSDTVLLEGKIDMWG-------VLIPIM---
AEO35601_Amm 82 N----------IH-FTLVADQDSDTVQLEGKIDMWG-------VLIPIM---
AEO36728_Amm 62 Q----------VE-IDFRAAHDSKTLRVRVLGAIGDM------PPQPFP---
Amc-759 70 K----------LE-VQF----------
ABI52703_Am 66 R----------LEFSAKNNNKYAVDVVVLVTIESL---------DRSHT---
ABI52704_Am 66 R----------LEFSAKNNNQYAVDVVVLVTIESL---------DRSHT---
ABI52707_Am 44 E----------LELS-LNGSTWAPDWHLPVRVSLFQ-------RKPKKW---
ABI52725_Am 60 R----------LRFGACYNATDTLQIYVYASVK----------QGNYT---
ABR23350_Op 73 T----------ME-VQFVSPIDTTSVYRSWYGCFGASRR-GRETRVRYGREV
ABR23463_Op 57 Q----------LRFTGMNRERYPLYLSVWADVRSIN--------HPGDS---
ABR23489_Op 57 H----------LEFAGRNHEAHPIDVFVWAHIQGIG--------HSEDS---
ACB70356_Oc 59 R----------VKFRSINNSRFTVGVGIHAYFNTTR-------RNVTTQ---
ACB70390_Oc 1 ----------GVYRSWFGCFGGSRRSGRETRVRYGREV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CAD68004_Ir_AL 85 -GIETDLCK-GTVDCP-----VIRD---GSTASPP----------S----------
ABU43149_IrML 95 -GFKSDACSNMGVECP-----LVAGKQYTAKSQLTMSPTF-PPTQAKAIFKGV-D---AA
AAP84098_Ir_ML 98 -GFKSDACSQMGVECP-----LVAGKQYLP----SLSSRC-PLHSRRLKQRPF-S---KV
AAY66768_Is 122 -EEVSFNFTNPDVDMP---LRVTPNETFK--VTVRLCRKF-LPYDFKLKIYVGTW----Q
AAY66573_Is 71 -EEVSFNFTNPDVDMP---IRVKENDTFK--ITARLCRKF-HPYDFKLKIFVGTW----Q
EEC00381_Is 97 -VPQPDACQ-SGVTCP-----VQPAGKYNYRGSFPIKPMY-PSTSLDIKWELLDD---KD
EEC03680_Is 88 -NFGRNACKKNGLTCP-----LESGKPYTLQSTLNVLSSF-PTVRGSRGVYMKGD---N-
EEC06676_Is 59 -GVEKDACRSGAVVCP-----VHKGKLFAGTISAYVYNFV-PSLTVTTTWKMVGA---QG
EEC07080_Is 91 -FNQKDGCKGSGLDCP-----LVAGRNYTVSRAVRVYRIY-PKVEILAVFEIRGN---DG
EEC07323_Is 121 -GFKSDACSNMGVECP-----LVAGKQYTAKSQLTMSPTF-PPTQAKAIFKGV-D---AG
EEC12523_Is 48 -INMVTWDSCHFVDVP---CTVKAGETFMGNVKVPIHKAFSAVSKIPGRVSVWTV----E
EEC13424_Is 62 -SLKKDGCREQGIRCP-----LEKNEKYVFTYSLLIYLL-FQLNTTAKLSLTGA---KG
EEC13776_Is 86 -GIETDLCN-GVIKCP-----VVKGQTYKGTIIFP----------
EEC16220_Is 25 -GFKSDACSQMGVECP-----LVAGKQYTAKSQLTMSPTF-PPTQAKAIFKGV-D---AA
EEC18444_Is 109 NVCNSTTSIDDNVRGSEESHGLRQGRLYRHSGAFLVKPFF-PKVQLNVTVYLYDK-SPQK
FD482838_Is ----------
TC55406_Is 98 -GFKSDACSQMGVECP-----LVAGKQYTAKSQLTMSPTF-PPTQAKAIFKGV-D---AA
TC56093_Is 108 -GFKSDACSNMGVECP-----LVAGKQYTAKSQLTMSPTF-PPTQAKAIFKGV-D---AG
ACF35546_Dv 107 -GFKTDACNFMGVSCP-----LKAGEKYTAKFDLTLSPTF-PPVAAKAVFKGQ-D---AA
ACF35538_Dv 93 -GFKTDACNFMGVSCP-----LKAGEKYTAKFDLTLSPTF-PPVAAKAVFKGQ-D---AA
Ra-2595 107 SICDSTASVDDDVKCSQADRGLREGHTYRHTGAFFVKPFF-PKVKVDVAIYVYDKEGPSK
CK186585_Rm 121 -GFKKNACTKSGLECP-----LEAGKQYTFSKQISVLPSF-PTLEIMAEVRLKAD---NG
CK187450_Rm 75 -AFKTDACNFMGVSCP-----LKAGDKYTAKFELAMSPTF-PPVAGKAVFKGQ-D---AA
Rm-1523 90 -IQSARSISCYYIDVP---CNVTKGEVFRGSVTLRILGAF-APGNLTYKIGVGHG----K
Rm-1718 88 -GFKKNACTKSGLECP-----LEAGKQYTFSKQISVLPSF-PTLEIMAEVRLKAD---NG
Rm-2126 108 SICDSTASVDDDVKCSQADRGLRGGHTYRHTGAFFVKPFF-PKVKVDVAVYVYDKEGPTK
Rm-22 42 -GFRKNACKNSGLTCP-----LEVGKSYTLQSTLNVLSSF-PSVDANVEWSMKGD---N-
Rm-3273 85 -GLDKDLCE-NMVKCP-----ISKGQTYSGVMEVYVPPFA-PAVKTHVSLKVVGD---KG
Rm-3607 92 -LPQSDACQ-SGVVCP-----IKAGASYTYRGSFPVRESY-PSISLDVKWELVDD---NG
Rm-4183 85 -GVEKDLCK-YALQCP-----VVKGNTYQGSIDYVPWFI-PSVKTTAQIKLVGD---EG
Rm-8994 85 -GMERDMCK-VVYHCP-----VVKGRTYNGTMTVHVPFYA-PQFEVNVQLKVIGD---KG
TC22814_Rm 99 -GFKKNACTKSGLECP-----LEAGKQYTFSKQISVLPSF-PTLEIMAEVRLKAD---NG
AEO32820_Amm 125 -GLESDLCK-GTIQCP-----VVKGQTYSGTIDVVPRLF-PPVKSTVQFRITGD---EG
AEO32826_Amm 109 -GLESDLCK-GTIQCP-----VVKGQTYSGTIDVVPRLF-PPVKSTVQFRITGD---EG
AEO33003_Amm 116 -GIEPNMCK-EVVKCP-----IKKGQTYRGTLVTPIPSIA-PAGETSLTLKVKGD---TG
AEO34342_Amm 93 -MPNDDGCKNSGIECP-----VKSGEKYMYVQEIEVKPSY-PKVSATIRWSLGDE---SG
AEO34818_Amm 85 -GLESDLCK-GTIQCP-----VVKGQTYSGIIEADVPWFV-PAVKSTVQFTITGD---KG
AEO35065_Amm 107 NICNATASVDDDVKCTQGNRGLRQGHLYQHTGAFFVKPFF-PKVKVNVAVYVYDKEGTNK
AEO35328_Amm 84 -MTDRDGCKGRGIQCP-----LRESAAYTFNYKLKVEPFY-PKVNTTAKLNLTGA---RG
AEO35404_Amm 91 -GFRKNACKNNGLSCP-----LEAGKSYTLQSTLNVLSSF-PSVDANVEWSMKGD---N-
AEO35600_Amm 113 -GLEKDMCK-GVVQCP-----VKKGETYTGTLVVSVPSFA-PSVESHVVLKIIGK---EG
AEO35601_Amm 113 -GLEKDMCK-GVVQCP-----VKKGETYTGTLVVSVPSFA-PSVESHVVLKIIGN---EG
AEO36728_Amm 94 -AFKTDACNFMGVSCP-----LKAGEKYTAKFELTMSPTF-PPVVGKAVIKGQ-D---AA
Amc-759 ----------
ABI52703_Am 96 -QYFRATCEDYNINFP---CRAEPNQEITGYMQIPLDKEY-KEGAATFTVHAY-------
ABI52704_Am 96 -QYFRASCQDYYINFP---CTAKPNEEITGYMQIPLDKEY-KEGAATFTVHAY-------
ABI52707_Am 75 -QHAELSCPERCTVHP---CRVKAARPFGVYLHPTVDEDF-KPGNALLNIEVGSQ----G
ABI52725_Am 88 -QMYRSTCNEAGTGYP---CSVRGQQNFTGYVHFILNDQL-EAGTRR-PDRMD-------
ABR23350_Op 113 NVCNVTASASGES-CTQN-HGLKQGVEYIQSGTFKVMQYF-PKVSVNVEIELYAKERRIV
ABR23463_Op 88 -FVYSSNCQSEWTNYP---CVAQPGRNVTGYVAIKIPSSF-KKSLTRIFFNVD-------
ABR23489_Op 88 -FYYSSGCHDDFTSYP---CIARPNESITGRLAIRLPKSF-KEGLARITFEVH-------
ACB70356_Oc 91 -WRIRTDCKTPGVHHQ---CKAAPDETVEGTVNFTILND--EDFLGKGPMRLDNF-----
ACB70390_Oc 29 NVCNATRSASGEL-CTET-QGLRQGVEYYQDGTFKVMQYF-PKVSVSVEIEVYTKERRAL

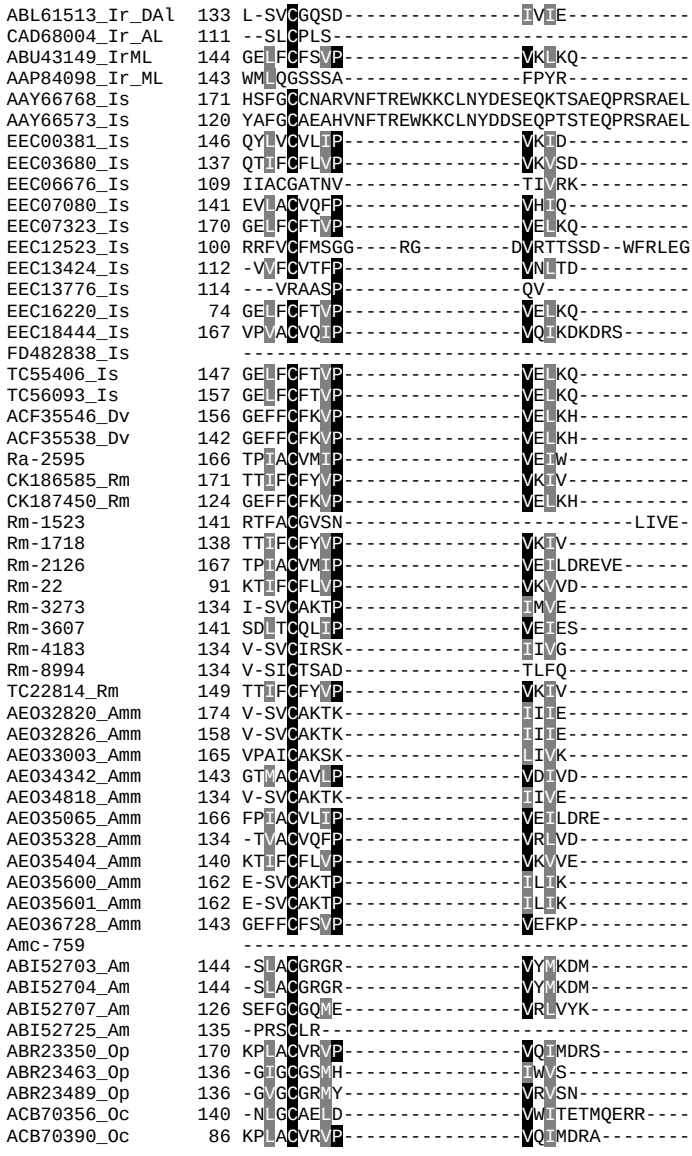

Fig. 21. Alignment of known tick ML proteins. Sequence were obtained from GenBank and from review by Francishetti et al. (2009). Abbreviated names of tick species are: *Ixodes ricinus* (Ir), *Ixodes scapularis* (Is), *Dermacentor variabilis* (Dv), *Rhipicephalus appendiculatus* (Ra), *Rhipicephalus microplus* (Rm), *Amblyomma maculatum* (Amm), *Amblyomma canajense* (Amc), *Argas monolakensis* (Am), *Ornithodoros parkeri* (Op), *Ornithodoros coriaceus* (Oc).

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