Ticks and Tick-borne Diseases 7 (2016) 565–572

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

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

Original article

Tick iron and heme metabolism – New target for an anti-tick intervention

Ondrej Hajdusek*, Radek Sima, Jan Perner, Gabriela Loosova, Adela Harcubova, Petr Kopacek

Institute of Parasitology, Biology Centre, Czech Academy of Sciences, Branisovska 31, 370 05 Ceske Budejovice, Czech Republic

ARTICLE INFO

Article history:
Received 8 September 2015
Received in revised form 12 January 2016
Accepted 15 January 2016
Available online 18 January 2016

Keywords:
Tick
Iron
Heme
RNAi
Vaccine

ABSTRACT

Ticks are blood-feeding parasites and vectors of serious human and animal diseases. *Ixodes ricinus* is a common tick in Europe, transmitting tick-borne encephalitis, Lyme borreliosis, anaplasmosis, or babesiosis. Immunization of hosts with recombinant tick proteins has, in theory, the potential to interfere with tick feeding and block transmission of pathogens from the tick to the host. However, the efficacy of tick antigens has, to date, not been fully sufficient to achieve this. We have focused on 11 *in silico* identified genes encoding proteins potentially involved in tick iron and heme metabolism. Quantitative real-time PCR (qRT-PCR) expression profiling was carried out to preferentially target proteins that are up-regulated during the blood meal. RNA interference (RNAi) was then used to score the relative importance of these genes in tick physiology. Finally, we performed vaccination screens to test the suitability of these proteins as vaccine candidates. These newly identified tick antigens have the potential to improve the available anti-tick vaccines.

1. Introduction

Ticks are blood-feeding ectoparasites transmitting viral, bacterial, and protozoal diseases to humans and animals (Hajdusek et al., 2013; Jongejan and Uilenberg, 2004). In Europe, Lyme borreliosis (LB) and tick-borne encephalitis are the most frequent human diseases transmitted by ticks (Mansfield et al., 2009; Rizzoli et al., 2011). Ticks and tick-borne diseases also greatly affect livestock, limiting production in many areas around the world. Protection against ticks and economic losses on animals are estimated to amount to billions of US dollars every year (Sonenshine and Roe, 2014). The castor bean tick (*Ixodes ricinus*) is commonly found in Europe and is closely related to the blacklegged tick *I. scapularis*, a vector of LB in the USA, and whose genomic sequence has been recently released (Lawson et al., 2009). Because of a high gene similarity (more than 95% of the nucleotide sequence), genomic information on this species can be directly applied to *I. ricinus* research.

Immunization with tick proteins to protect hosts against tick feeding or transmission of pathogens is a challenge not only for livestock production, but is also important for human health (Merino et al., 2013; Moyer, 2015). Because vaccine production is not as difficult or expensive as production of acaricides (Bowman and Nuttall, 2008), scientists are encouraged to find suitable tick antigens that could be used for vaccine development. The first commercialized vaccine (TickGUARD, Gavac), which protected cattle from *Rhipicephalus microplus*, was based on the tick midgut protein BM86 (Willadsen et al., 1995). This vaccine also interfered with transmission of babesiosis (de la Fuente et al., 2007). However, the BM86 vaccine is specific against cattle tick (*Rhipicephalus* spp.) infestations only with limited efficacy against other tick species (de la Fuente and Kocan, 2003). Protection based solely on vaccination therefore requires the identification of new, more efficient antigens.

RNA interference (RNAi) technologies have raised new options for screening tick genes as new vaccine candidates. Using RNAi we have previously described the basic pathway of iron metabolism in *I. ricinus* and identified a crucial protein, ferritin2 (FER2), with a novel function (Hajdusek et al., 2009). Immunization of cattle or rabbits with recombinant FER2 dramatically reduced tick feeding, tick weight after feeding, and the fertility of various tick species. The protective efficacies were similar to those obtained with a commercial vaccine based on BM86 (Hajdusek et al., 2010). Recently, vaccinations with recombinant FER2 and ferritin1 (FER1) were shown to reduce tick feeding and oviposition, and hatching, respectively, in the hard tick *Haemaphysalis longicornis* (Galay et al., 2014), reinforcing the potential of tick ferritins as universal vaccination antigens applicable against multiple tick species.

* Corresponding author.
E-mail addresses: hajdus@paru.cas.cz (O. Hajdusek), sima@paru.cas.cz (R. Sima), perner@paru.cas.cz (J. Perner), gabi@paru.cas.cz (G. Loosova), harcubova@paru.cas.cz (A. Harcubova), kopajz@paru.cas.cz (P. Kopacek).

http://dx.doi.org/10.1016/j.ttbdis.2016.01.006
1877-959X/

Iron is an indispensable inorganic element in most organisms. Because of its redox properties, iron serves as an electron donor and acceptor in various metabolic processes. Iron or iron-sulfur clusters are core components of many enzymes involved e.g., in the respiratory chain of mitochondria or DNA biosynthesis. Heme, a prosthetic group that holds an iron atom at the center of a porphyrin ring, is a key component of hemoproteins e.g., for transport of oxygen, electron transfer in mitochondria, or defense mechanisms against oxidative stress. Importantly, iron participates in the formation of toxic radicals that cause substantial damage to proteins, lipids, and DNA. For this reason, iron and heme homeostasis is maintained in every organism by an orchestrated set of sophisticated proteins handling their uptake, utilization, transport, and storage (Hamza and Dailey, 2012; Hentze et al., 2004).

Here, we screened for new vaccine candidates in the tick iron and heme metabolic pathways. The genes identified in the available tick sequence databases were initially molecularly characterized in silico and their expression profiles were determined in different tick stages and tissues by relative quantitative real-time PCR (qRT-PCR). RNAi was then used to score their importance during and after tick feeding. Rabbits were vaccinated with recombinant proteins and potential anti-tick antigens were evaluated. From this novel investigation of iron and heme metabolism in ticks we identified several genes that effected tick feeding, oviposition, and hatching. Production of new vaccine antigens that interfere with tick iron and heme metabolism could help in strategies to fight ticks and tick-transmitted diseases.

2. Materials and methods

2.1. Ticks

Adult females and males of *I. ricinus* were collected by flagging around Ceske Budejovice, Czech Republic and were used for RNAi (guinea pigs) and vaccination (rabbits) experiments. Ticks were maintained in wet chambers with a humidity of about 95%, temperature 24 °C, and day/night period set to 15/9 h. To obtain tick developmental stages for qRT-PCR experiments, females were fed on laboratory guinea pigs in the presence of males. Larvae were fed on guinea pigs, nymphs were fed on guinea pigs or rabbits. Molted adult females (pathogen free) were used for pathogen injection/feeding experiments (Urbanova et al., 2015). All laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 021/2012.

2.2. Identification and characterization of genes

To identify tick genes possibly involved in iron and heme metabolism, we performed a BLAST search at NCBI (http://www.ncbi.nlm.nih.gov/) using homologous arthropod gene sequences as baits (Supplementary Table 1). Full sequences of the genes (from *I. scapularis* or *I. ricinus*) were screened in silico for putative signal sequences (SignalP; www.cbs.dtu.dk/services/SignalP/), cellular localizations (PSORT; http://psort.hgc.jp/form.html), the presence of transmembrane motifs (TMHMM; http://www.cbs.dtu.dk/services/TMHMM/), and glycophosphatidylinositol anchors (big-PI; http://mendel.imp.ac.at/sat/gpi/gpi_server.html). The phylogenetic analysis in Fig. 4A was carried out as described previously (Sojka et al., 2007).

2.3. Relative expression profiling by quantitative real-time PCR (qRT-PCR)

Total RNA, isolated from *I. ricinus* developmental stages, tissues, and unfed adults injected or fed with different pathogens, was prepared as described previously (Urbanova et al., 2014, 2015). All RNA samples were prepared in biological triplicates, transcribed into cDNA (Roche), and analyzed by quantitative real-time PCR using a LightCycler 480 (Roche) and SYBR green chemistry as described previously (Urbanova et al., 2015). For the primers used in qRT-PCR, see Supplementary Table 2. Relative expression was calculated using the mathematical model of Pfaffl (Pfaffl, 2001) and normalized to *elongation factor1* (Nijhof et al., 2009). RT-PCR profiling shown in Fig. 2B was performed as described previously (Hajdusek et al., 2009).

2.4. Impact of gene silencing on tick feeding and development

234–521 bp (for details see Supplemental Table 2) gene-specific double-stranded RNAs (dsRNA) were synthesized and injected into adult females of *I. ricinus* as described previously (Hajdusek et al., 2009). Injected ticks (25 per group) were mixed with an equal number of males, placed in cylinders on the backs of guinea pigs and allowed to feed naturally until repletion. After feeding, ticks were visually checked, weighed, and put into separate vials to evaluate oviposition and hatching. Ticks injected with dsRNA against green-fluorescence protein (GFP) served as a control in all experiments. Gene silencing was checked by qRT-PCR in midgut, salivary glands, and ovaries of half-fed ticks (pool of tissues dissected from five females). In Fig. 4B, homogenates of RNAi-silenced tick tissues were assayed by Western Blot analysis using anti-FER1 antibody (Kopacek et al., 2003) to evaluate involvement of *transferrin2* knock-down (KD) in the transport of iron from the tick gut to peripheral tissues, as described previously (Hajdusek et al., 2009).

2.5. Effect of vaccination on tick feeding and development

Full-length (whole coding sequence without signal peptides) or partial gene sequences were amplified from *I. ricinus* cDNA (for details see Supplemental Table 2), cloned into the expression vector pET100 (Invitrogen, containing N-terminal His-tag), expressed in *E. coli* BL21 (Invitrogen), purified, and refolded (Hajdusek et al., 2010). To immunize rabbits, 1 ml of the recombinant protein (100 μg/ml) was mixed 1:1 with Freund's adjuvant and injected subcutaneously in three doses (weeks 1, 3, and 6) as described previously (Hajdusek et al., 2010). Negative controls were injected with adjuvant/saline (50 mM Tris pH 9, 150 mM NaCl). Two weeks after the last immunization, a sample of rabbit blood was taken from the ear to check development of specific antibodies before and after vaccination by Western Blot analysis. Rabbits were then infested with 25 *I. ricinus* pairs per animal, in glued cylinders. Engorged ticks were visually checked, weighed, and put into separate vials to evaluate ensuing oviposition and hatching.

3. Results

3.1. Identification and molecular characterization of proteins possibly involved in iron and heme metabolism

We have screened available *I. ricinus* and *I. scapularis* sequences accessible in GenBank to identify tick proteins that are likely to participate in the maintenance of iron and heme homeostasis. In addition to three previously identified iron metabolism genes, *ferritin1*, *ferritin2*, and *iron-regulatory protein1* (*irp1*) (Hajdusek et al., 2009), we identified two new genes of iron metabolism and six genes of heme metabolism with clear sequence homologies to other invertebrates (Table 1). Our in silico analysis revealed that divalent metal transporter1 (DMT1) was a 563 amino acids (aa) protein containing 10 transmembrane motifs (TMM) with predicted localization to peroxisomes or the plasma membrane. Transferrin2 (TF2) was a 793 aa protein containing a predicted signal peptide,