Ticks and Tick-borne Diseases xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

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

Multienzyme degradation of host serum albumin in ticks

Daniel Sojka[a,*,1], Jana Pytelková[b,1], Jan Perner[a,c], Martin Horn[b], Jitka Konvičková[a,c], Jana Schrenková[a,c], Michael Mareš[b], Petr Kopáček[a]

[a] Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Branisovska 31, 373 11 Ceske Budejovice, Czech Republic
[b] Institute of Chemistry and Biochemistry, Czech Academy of Sciences, Flemingovo nam. 2, 166 10 Prague, Czech Republic
[c] Faculty of Science, University of South Bohemia, Branisovska 31, 373 11 Ceske Budejovice, Czech Republic

ARTICLE INFO

Article history:
Received 7 September 2015
Received in revised form
25 November 2015
Accepted 14 December 2015
Available online xxx

Keywords:
Albumin digestion
Tick
Proteolysis
Gut

ABSTRACT

Host blood proteins, represented mainly by hemoglobin and serum albumin, serve as the ultimate source of amino acids needed for *de novo* protein synthesis during tick development and reproduction. While uptake and processing of hemoglobin by tick gut cells have been studied in detail, molecular mechanisms of host serum albumin degradation remain unknown. In this work, we have used artificial membrane feeding of *Ixodes ricinus* females on a hemoglobin-free diet in order to characterize the proteolytic machinery involved in albuminolysis. Morphological comparisons of ticks fed on whole blood (BF) and serum (SF) at microscopic and ultrastructural levels showed that albumin and hemoglobin have different trafficking routes in tick gut cells. Analysis *in vitro* with selective inhibitors demonstrated that albumin is degraded at an acidic pH by a network of cysteine and aspartic peptidases with predominant involvement of cysteine cathepsins having endo- and exopeptidase activities. The cleavage map of albumin and the roles of individual peptidases in albumin degradation were determined. These results indicate that the albuminolytic pathway is controlled by the same proteolytic system that is responsible for hemoglobinolysis. This was further supported by the overall similarity of gut peptidase profiles in SF and BF ticks at the transcriptional and enzymatic activity levels. In conclusion, our work provides evidence that although hemoglobin and albumin are transported differentially during heterophagy they are digested by a common multienzyme proteolytic network. This central digestive system, critical for successful blood feeding in tick females, thus represents a valuable target for novel anti-tick interventions.

Introduction

Blood feeding (hematophagy) is an essential physiological process for ticks since host blood serves as their ultimate source of energy and nutrients utilized in their development and reproduction. Processing of blood in ticks differs greatly from that in other hemathophagous arthropods. In blood-sucking insects, blood protein digestion proceeds rapidly in the gut lumen and is carried out mainly by alkaline serine proteases (Briegel and Lea, 1975). In contrast, blood digestion in ticks is a much slower process that occurs inside the acidic vesicles of tick gut cells (Grandjean and Aeschlimann, 1973). Notably, the virtual absence of proteolytic enzymes in the gut contents creates a highly favorable environment for the survival of ingested microorganisms (Sonenshine and Roe, 2014) and represents one of the key factors making ticks potent vectors of an enormous number of human and domestic animal diseases.

Hard ticks (Ixodidae) feed only once per life-stage (larvae, nymph, adult female or male) and females die several days after laying down a large clutch of eggs (Coons and Alberti, 1999). Feeding of females lasts for several days and consists of a slow feeding period, taking approximately 6–9 days, followed by a rapid engorgement occurring 12–24 h prior to detachment from hosts and accounting for about two-thirds of the total blood volume ingested during the slow feeding period. Only mated females can proceed to rapid engorgement indicating a yet uncharacterized physiological control mechanism.

Albumin and hemoglobin serve as the main sources of amino acids for blood-feeding organisms since these proteins account for more than 80% of the total protein content of vertebrate blood. Digestive gut cells take up blood proteins by heterophagy (Sonenshine and Roe, 2014), which in ticks is comprised of at least two distinct endocytic mechanisms. As demonstrated by the tracking of fluorescent hemoglobin and albumin in a primary gut cell line from *Rhipicephalus (Boophilus) microplus* (Lara et al., 2005), the two

* Corresponding author. Tel.: +420 387775453; fax: +420 385310388.
E-mail address: sojkadan@gmail.com (D. Sojka).
[1] These authors share the first authorship.

http://dx.doi.org/10.1016/j.ttbdis.2015.12.014
1877-959X/

major blood proteins, albumin and hemoglobin, are not handled the same way by the tick gut digestive cells. While albumin is taken up by fluid-phase endocytosis and is directed into a population of small acidic vesicles, hemoglobin is recognized by specific cell-surface receptor(s), which target it to a population of large endosomal vesicles (Lara et al., 2005). This is supported by early ultrastructural studies on tick gut epithelial cells that describe coated pits in the midgut epithelium (Coons et al., 1986). Receptor-mediated endocytosis and a separate processing pathway for hemoglobin most likely evolved as a detoxification mechanism against the potentially dangerous heme that is released upon digestion of hemoglobin (Graca-Souza et al., 2006; Lara et al., 2003).

Existing knowledge on tick digestive proteolytic enzymes began to be assembled from the 1980s to 1990s. First reports included individually characterized 'hemoglobinases' of various classes from different tick species, providing a rather fragmented picture of the tick hemoglobinolytic system (reviewed in (Sojka et al., 2013)). Focusing on one tick species, the major European Lyme disease vector, *Ixodes ricinus*, at a single well-defined life stage (partially-engorged female), we have previously employed a number of reverse genetic and proteomic approaches to globally profile the hemoglobinolytic machinery in the gut (Horn et al., 2009; Sojka et al., 2008, 2013). A mechanistic model for the uptake and proteolytic degradation of hemoglobin degradation in the digestive cells of *I. ricinus* was presented recently (Sojka et al., 2013). Briefly, endopeptidases, aspartic cathepsin D, supported by cysteine cathepsin L and legumain, are responsible for the primary hemoglobin cleavage events. Endopeptidolytic activity of cathepsin B participates in the production of smaller secondary fragments. The pool of peptide fragments released by these endopeptidases is degraded by the action of exopeptidases through the dipeptidase activities of cathepsins B and C (Horn et al., 2009. The unbound heme moiety forms aggregates that accumulate in the hemosomes (Lara et al., 2003).

In the present study we fill some of the gaps in knowledge on the two separate trafficking pathways of hemoglobin and serum albumin by mapping tick gut cell peptidases responsible for the digestion of serum albumin. Our primary task was to determine whether the component peptidases of the multienzyme hemoglobinolytic network were also involved in the degradation of host albumin. To address these fundamental questions we exploited our recently developed protocol for tick artificial membrane feeding on whole blood and hemoglobin-free serum (Perner et al, submitted manuscript). This powerful tool, combined with molecular approaches previously employed in the mapping of digestive hemoglobinolysis in *I. ricinus*, enabled a comprehensive analysis of albumin degradation in the same species and a comparison of both proteolytic pathways.

Materials and methods

Tick collection, maintenance and feeding

Adult *I. ricinus* females and males originating from the pathogen-free colony of Institute of Parasitology, BC CAS were used throughout the study. Ticks were maintained in glass vials at 24 °C, 95% humidity, and 15:9-h day/night regime. For natural feeding, 25 females were allowed to feed on the backs of guinea pigs, in the presence of 25 males, until full engorgement, or were forcibly removed from the host at the specified feeding phase. All laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 095/2012. For artificial membrane feeding, the procedure developed by Krober and Guerin (2007) was adapted as described elsewhere (Perner et al., submitted manuscript). Briefly, bovine blood, acquired from the local slaughterhouse, was manually defibrinated by stirring and the remaining clot was removed using a sterile strainer. The hemoglobin-free serum was prepared by centrifugation at 2500 × *g* for 10 min at 4 °C and the supernatant was centrifuged again at 10,000 × *g* for 10 min at 4 °C. Both the whole blood and serum were supplemented with filter-sterilized glucose to a final concentration of 2 g/l. Immediately prior to feeding, both blood and serum meals were supplemented with adenosine triphosphate (1 mM) and gentamicin (5 μg/ml), 3.1 ml of the meal was pipetted into each feeding unit adapted for a 6-well cultivation plate and regularly exchanged at intervals of 12 h. Fifteen *I. ricinus* females were placed into one feeding unit lined with a thin (80–120 μm) silicone membrane, previously pre-treated with a bovine hair extract (Krober and Guerin, 2007). An equal number of males were added to the feeding units 24 h post-attachment of females to the membrane. Females were allowed to feed until full engorgement or removed from the membrane at the specified time of feeding.

Preparation of tick gut homogenates for biochemical and proteomic analyses

Twelve feeding units were prepared for two groups of ninety *I. ricinus* females, either fed on whole blood (blood-fed, BF) or serum (serum-fed, SF). In both groups, about 50% of the females attached and successfully fed on the applied diet. Partially-engorged females were removed from the membrane, individually weighed and gut tissues (from each group) were dissected under a stereo microscope with LED illumination (Stemi DV4, Zeiss). Dissected gut tissues were washed in sterile PBS to remove excess blood from the gut lumen without disruption of gut epithelial cells. Pools of gut tissues were prepared from each BF and SF group and stored at −80 °C until homogenization. Gut tissue extracts (400 μg of protein/ml) were prepared by homogenization of the pooled gut tissue in 50 mM Na-acetate, pH 4.5 and 1% CHAPS on ice. The extract was cleared by centrifugation (16,000 × *g*, 10 min, 4 °C), filtered through an Ultrafree-MC 0.22 μm filter (Millipore) and stored at −80 °C.

Tick gut dissection for RNA isolation and reverse transcription

For each specified time point, guts from nine BF/SF *I. ricinus* females were dissected and divided into three independent biological replicates (3 guts per sample). Sterile diethyl pyrocarbonate (DEPC)-treated PBS was used for dissection and washing of gut tissues. Total RNA was isolated from dissected guts using a NucleoSpin® RNA II kit (Macherey-Nagel). The quantity and quality of isolated total RNA was verified using a NanoDropTM 1000 spectrophotometer and 1% agarose gel electrophoresis, respectively. Isolated RNA samples were stored at −80 °C prior to cDNA synthesis. Single-stranded cDNA was reverse-transcribed from 0.5 μg of total RNA using the Transcriptor High-Fidelity cDNA Synthesis Kit (Roche). For subsequent applications, cDNA was diluted 20 times in nuclease-free water.

Microscopy techniques

Partially-engorged *I. ricinus* females were dissected in 10 mM Hepes buffer, pH 7.4. Tick gut tissue was transferred to a freshly prepared fixative (4% formaldehyde, 0.1% glutaraldehyde in 10 mM Hepes buffer, pH 7.4) and incubated at room temperature for 1 h. Samples were further washed with 0.1 M Na-phosphate buffer (PB), pH 7.4, containing 4% glucose. Tissues were transferred to pre-cooled cryogenic vials and dehydrated in acetone at −10 °C (30%, 50%, 70%, 80%, 90% and 95% for 30 min at each step). The samples were rinsed three times in anhydrous acetone at room

Please cite this article in press as: Sojka, D., et al., Multienzyme degradation of host serum albumin in ticks. Ticks Tick-borne Dis. (2015), http://dx.doi.org/10.1016/j.ttbdis.2015.12.014