

Immunoglobulin G binding protein (IGBP) from *Rhipicephalus haemaphysaloides*: identification, expression, and binding specificity

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Abstract As the second most important human ectoparasite, ranked only after mosquitoes, the tick threatens the development of husbandry and even the health of humans worldwide. Immunoglobulin G binding proteins (IGBPs) are considered to be the major factors used by ticks to evade the host immune system and the damage caused by host antibodies. In this study, an IGBP-MB homologue was identified in the tick *Rhipicephalus haemaphysaloides*, which was predominantly detected in the salivary glands and hemolymph of male ticks. Recombinant IGBP (rIGBP/His) displayed significant binding activity to IgGs from rabbits and pigs, and bound to the F(ab)² but not the Fc fragment of rabbit IgG. Although the silencing of IGBP expression in ticks had no obvious effect on their blood-feeding and subsequent oviposition, antibodies raised to rIGBP/GST reduced the replete body weight (218.9±20 mg in the control group vs. 142.5±43.3 mg in the test group, $P<0.05$ by Student's *t* test) and increased the mortality of the ticks. This study extends our understanding of the immunoevasive function of IGBPs and is a step towards the development of a vaccine against ticks.

Keywords Immunoglobulin G binding protein (IGBP) · *Rhipicephalus haemaphysaloides* · Binding specificity

Introduction

It is well known that various viruses, bacteria, and parasites are transmitted by vectors and that the best way to control these pathogens is to eliminate their vectors, which include mosquitoes and ticks. The tick ranks second only to the mosquito in its importance in the transmission of infectious agents (Sonenshine 1993) including 126 kinds of viruses, 14 kinds of bacteria, 18 kinds of spirochetes, 20 kinds of rickettsia, and 26 kinds of protozoa. At present, chemicals are usually used to control ticks worldwide, which has led to a resistance in this ectoparasite (George et al. 2004), and the chemical residues in milk and meat products threaten the health of humans. Biocontrol agents have not been used because several problems are still unresolved (e.g., environmental stability, ability to initiate infection at low humidity, and potential to damage to other species (Tavassoli et al. 2012)). The development of the first commercial vaccine, Bm86, a successful “concealed antigen” against *Rhipicephalus microplus* (Willadsen 2004), began the functional identification of appropriate target molecules and the development of candidate vaccines for ticks. However, to date, there is no significantly effective vaccine against ticks, except for several species, such as *Rhipicephalus (Boophilus)* and *Hyalomma* (de la Fuente et al. 2007; Fragoso et al. 1998; Rodriguez-Valle et al. 2012). It has been reported that antibodies mediate an immunoreaction that damages the cells of parasites or blocks the blood-feeding of ectoparasites (Singh and Girschick 2003). However, the immunoglobulin G of mammalian hosts was shown to pass through the midgut barriers of *Rhipicephalus appendiculatus* into the hemolymph

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and was excreted via the saliva back into the host during feeding (Wang and Nuttall 1999). The immunoglobulin G binding proteins (IGBPs) in the tick hemolymph and salivary glands are thought to be responsible for this excretion (Wang and Nuttall 1999).

In 1994, Wang and Nuttall detected the first IGBP from *R. appendiculatus*. Later, IGBPs were found in other tick species, including *Amblyomma variegatum* and *Ixodes hexagonus*, of different molecular sizes and subtypes (Wang and Nuttall 1995a). In the same year, Wang and Nuttall (1995b) isolated three kinds of IGBPs from the salivary glands of male *R. appendiculatus*, designated IGBP-MA, IGBP-MB, and IGBP-MC, with different molecular sizes and different binding activities. IGBP-MB showed 50 % homology to IGBP-MC, but both displayed low identity to IGBP-MA. IGBP-MC bound to guinea pig IgG (on which species the ticks were fed) as well as human and bovine IgG, whereas IGBP-MA and possibly IGBP-MB only bind to guinea pig IgG (Wang and Nuttall 1995b). These IGBPs expressed in male ticks are suggested to help female ticks feed on blood and remove IgG from the tick itself to evade the damage caused by the antibody-mediated immunoreaction of the host. In this context, IGBPs are critical molecules and potential candidate vaccines, which can circumvent the strategies ticks use to protect themselves from the host's antibodies during feeding. However, IGBPs have not been completely characterized and the mechanism of their role in the blood-sucking process is not fully understood.

In this study, we isolated an IGBP from the tick *Rhipicephalus haemaphysaloides*, which is distributed widely in Asia. The expression profile of IGBP, the binding activity of its recombinant protein for the IgGs of several host species, and the binding site on IgG were investigated, together with the dynamics of the host IgG–IGBP interaction in male ticks during feeding.

Materials and methods

Ticks and tissue preparation

The Hubei strain of *R. haemaphysaloides* was maintained on blood meals on the ears of New Zealand white rabbits at the Chinese Academy of Agricultural Sciences (Shanghai, China) (Zhou et al. 2006a). This work was approved by the Research Ethics Review Committee of the Chinese Academy of Agricultural Sciences (approval no. SYXK[SH2011-0116]). To obtain different tissues, 4-day-fed or unfed adult ticks were dissected as described elsewhere (Mulenga et al. 1999). In brief, after the surface was cleaned simply with 75 % ethanol, the dorsal cuticle of the tick was removed with a pair of soft-tissue

forceps under a dissection light microscope. Then, 10 μ l of autoclaved ice-cold phosphate-buffered saline (PBS; pH 7.4) was added to the cavity to collect the hemolymph. The salivary glands and midgut were then separated with 18-gauge needles. The tissues were placed in 1.5-ml microcentrifuge tubes with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) or PBS and stored at -80°C until use. For total RNA isolation, the whole ticks (fed or unfed) were ground in liquid nitrogen and kept in TRIzol Reagent until use.

Total RNA isolation and cloning the full-length gene by the rapid amplification of cDNA ends (RACE)

Total RNA was isolated from the samples stored in TRIzol, according to the manufacturer's instructions, and the RNA isolated from the 4-day-fed female adult ticks was used in a RACE reaction. From a subtracted salivary-gland library (cDNA from partially fed female ticks as the tester and cDNA from unfed female ticks as the driver) constructed previously (Xiang et al. 2009), the expressed sequence tags (ESTs) of IGBP were selected to design gene-specific primers (GSPs, shown in Table 1). To obtain the 3'-end fragments, nested polymerase chain reaction (PCR) amplification was performed with the 3'-RACE System (Invitrogen) using the 3-GSP and 3-nested-GSP primers, and the amplified PCR product was cloned with routine methods into the pEGM-T vector for sequencing. The 5-GSP1, 5-GSP2, and 5-nested-GSP primers used in 5' RACE were designed based on the results of 3'RACE and the ESTs. The final full-length sequence of *IGBP* was analyzed for homology in GenBank with BLAST, for signal peptide prediction using the SignalP software (<http://www.cbs.dtu.dk/services/SignalP/>) and for domain prediction with the SMART software (<http://smart.embl-heidelberg.de/>).

Expression and purification of rIGBP protein

The rIGBP protein was expressed in both prokaryotic and eukaryotic cells. To obtain a glutathione *S*-transferase (GST)-rIGBP fusion protein (rIGBP/GST), the open reading frame (ORF) of the *IGBP* gene with no signal peptide sequence was amplified with rIGBP/GST-F and rIGBP/GST-R (shown in Table 1), and subcloned into the pGEX-4T-1 expression vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The positive clone was confirmed by sequencing and transfected into *Escherichia coli* strain BL21(DE3) (Amersham Pharmacia Biotech). Soluble rIGBP/GST was then purified with glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech), according to the manufacturer's instructions, and the protein concentration was determined with sodium dodecyl sulfate

Table 1 Primers used in RACE, sequencing, recombinant protein expression, real-time PCR amplification, and RNA interference

Primers	Sequences
3-GSP	5'-CCACATCGTGCCATGCATTGAC-3'
3-Nested-GSP	5'-GGCAATGGAACAGAGCCCAATAAC-3'
5-GSP1	5'-CCGTTGCCAATGTGTTTG-3'
5-GSP2	5'-CTTTCTCCGCCACCGCCGAGAGATC-3'
5-Nested-GSP	5'-CAGCAGCACGGCGACGATGGCGAAC-3'
rIGBP/GST-F	5'-GTAGTCGACAAGAAAGCCCCGTGCAGTA-3'
rIGBP/GST-R	5'-GATGCGGCCGCCTAGTACCATTCCCTTCGCTT-3'
rIGBP/His-F	5'-GTCACTAGTAGAAAGCCCCGTGCAGTA-3'
rIGBP/His-R	5'-GAGAAGCTTCTAGTACCATTCCCTTCGTTGG-3'
qP-IGBP-F1	5'-TGAGATTGGCTTTGTTTCACGC-3'
qP-IGBP-R1	5'-GGGCACTGGGCATTGTTATT-3'
Actin-F	5'-ATGTGTGACGACGAGGAGGTTGCC-3'
Actin-R	5'-TTAGAAGCACTTGCCTGGACAAT-3'
IGBPi-243-U1	5'-GGATCCTAATACGACTCACTATAGGCCATTTT TTTGTGTCCTTAGACCACAG-3'
IGBPi-243-D1	5'-CGTGAACAAAGCCAATCTCA-3'
IGBPi-243-U2	5'-GGATCCTAATACGACTCACTATAGGCGTGA ACAAAGCCAATCTCA-3'
IGBPi-243-D2	5'-CCATTTTTTTGTGTCCTTAGACCACAG-3'
IGBPi-667-U1	5'-GGATCCTAATACGACTCACTATAGGTGAGA TTGGCTTTGTTTCACG-3'
IGBPi-667-D1	5'-GCTTATATATCGACCTTATCATC-3'
IGBPi-667-U2	5'-GGATCCTAATACGACTCACTATAGGGCTTATA TATCGACCTTATCATC-3'
IGBPi-667-D2	5'-TGAGATTGGCTTTGTTTCACG-3'
qP-IGBP-F2	5'-CGTCTACGACGCAATTCTCA-3'
qP-IGBP-R2	5'-AGAGGTCGTACACGCAGC-3'
Luci-U1	5'-GGATCCTAATACGACTCACTATAGGGCTTC CATCTCCAGGGATACG-3'
Luci-D1	5'-CGTCCACAAACACAACCTCCTCC-3'
Luci-U2	5'-GCTTCCATCTCCAGGGATACG-3'
Luci-D2	5'-GGATCCTAATACGACTCACTATAGGCGTCC ACAAACACAACCTCCTCC-3'
EF-1a-F	5'-CGTCTACAAGATTGGTGGCATT-3'
EF-1a-R	5'-CTCAGTGGTCAGGTTGGCAG-3'

polymerase gel electrophoresis. Purified rIGBP/GST was then used to prepare antiserum in mice. To generate His-tagged rIGBP, the amplified product of *IGBP* generated with primers rIGBP/His-F and rIGBP/His-R (shown in Table 1) was subcloned into the pFastBac HTa vector (Invitrogen). The positive clone was identified, extracted, and co-transfected with bacmid into Sf9 cells (cultured in Sf900-II SFM (Invitrogen) with 5 % fetal bovine serum) using Cellfectin II Reagent (Invitrogen). The rIGBP/His in the culture supernatant was checked by immunoblotting

and then purified with Ni-NTA His-Bind Resin (Novagen, Madison, WI, USA).

Investigation of IGBP expression in ticks with real-time PCR and immunoblotting

To check the mRNA expression of IGBP in *R. haemaphysaloides*, total RNAs isolated from eggs, larvae, nymphs, and adults were subjected to reverse transcription polymerase chain reaction (RT-PCR). The RNA from the tick midgut, salivary gland, and hemolymph separated from 4-day-fed adult ticks (as described in sections above) were subjected to reverse transcription (RT) with a PrimeScript RT Reagent Kit (TaKaRa, Liaoning, China) and subsequent real-time PCR with the qP-IGBP-F1 and qP-IGBP-R1 primers (Table 1) using the SYBR Premix Ex Taq™ kit (TaKaRa). β -Actin (GenBank accession no. HY140790) was used as the internal control. RT was performed at 37 °C for 1 h and then stopped by incubation at 85 °C for 10 s. Real-time PCR was carried out at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. It was performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq™ kit (TaKaRa). To detect the native protein in the tick, mice were immunized three times with purified rIGBP/GST (500 μ g per time) to prepare antisera, as described previously (Gong et al. 2008). The tissues collected above were sonicated and subjected to immunoblotting using anti-rIGBP/GST antibody (diluted 1:1,000 in PBS with 5 % skimmed milk and 0.1 % Tween) as the primary antibody. Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody diluted 1:1,000 was used as the secondary antibody.

Assay of rIGBP/His binding activity

The binding activity of purified rIGBP/His to IgGs was evaluated with an enzyme-linked immunosorbent assay (ELISA). In brief, rIGBP/His was incubated in a 96-well plate (0.5 μ g/well), and different IgGs purified from human, rabbit, pig, mouse, dog, and goat (Shanghai Ding Guo Biotech Co. Ltd, Shanghai, China) were individually reacted with the recombinant protein. Antibodies directed against the different IgGs (Bethyl, Montgomery, TX, USA) were used to detect the binding activity, and optical density at 415 nm (OD_{415}) was measured on an ELx800 Absorbance Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). To determine the binding site on IgG for rIGBP/His, rIGBP/His in a 96-well plate was reacted separately with the rabbit F(ab')₂ (1 μ g/well) and Fc (1 μ g/well, Rockland, PA, USA) fragments, and then the binding activity was examined with an ELISA, as described above.

Monitoring the dynamic changes in host IgG and IGBP in ticks during blood-feeding

A New Zealand white rabbit was immunized three times with bovine serum albumin (BSA) at a dose of 600 µg the first time, 400 µg the second time, and 300 µg the third time to prepare anti-BSA antiserum. The antiserum was collected 10 days after the last inoculation. Sixty pairs of *R. haemaphysaloides* ticks (males and females) were then fed on the two ears (30 pairs/ear) of the rabbit. During the blood-feeding period, six pairs of ticks were detached by hand each day, from which the hemolymph, midguts, and salivary glands were dissected, sonicated, and used separately as the male and female samples. To investigate the distribution of IgG in the different tissues of the ticks, 5 µg/well BSA was incubated in a 96-well plate and then the collected tissues (which were assumed to contain the host IgG) were used as the primary antibody to react with BSA, followed by reaction with an HRP-conjugated goat anti-rabbit antibody (Bethyl). The collected organs were also subjected to immunoblotting to check the IGBP distribution and changes, using anti-rIGBP/GST antiserum produced in mice as the primary antibody. Immunoblotting was performed as described in sections above.

RNA interference of IGBP expression in male ticks

cDNA derived from *IGBP* and cloned into the pMD-18-T vector was amplified by PCR using oligonucleotides that both included the T7 promoter sequence at the 5'-end. The PCR products were gel-purified to synthesize double-stranded RNA (dsRNA) by in vitro transcription with T7 RNA polymerase, according to the manufacturer's protocol (T7 RiboMAX™ Express RNAi System, Promega, Madison, WI, USA). To achieve a good interference effect, two parts of the dsRNA fragments were synthesized, corresponding to nucleotides 1–243 and 243–667 of *IGBP*, with four pairs of primers (IGBPi-243-U1 to IGBPi-667-D2, Table 1). The two kinds of synthesized dsRNAs were mixed 1:1, according to their copy numbers. The mixed dsRNA was injected as described previously (Zhou et al. 2006b). Because IGBP is mainly detected in male ticks, 1 µg of the mixed IGBP dsRNAs per tick was microinjected into 120 unfed male ticks. The injected ticks were allowed to rest for 24 h at 25 °C, and the surviving ticks were then fed, together with an equal number of untreated females, on the ears of two rabbits, with 30 pairs of ticks per ear. dsRNAs encoding the luciferase gene were synthesized (Luci-U1, Luci-D1, Luci-U2, and Luci-D2 were used as primers; Table 1) and used as the negative control. To investigate the effects of RNA interference, 5-day-fed and 13-day-fed male ticks were detached by hand from the rabbit ears and subjected to real-time PCR with primers Qp-IGBP-F2 and Qp-IGBP-R2 (Table 1) and to

immunoblotting analysis to check the protein product of IGBP. Elongation factor-1 alpha was amplified as the internal control in the real-time PCR, with primers EF-1a-F and EF-1a-R (Table 1). The attachment rate of the ticks 24 h after inoculation, the engorgement rate, the engorged body weight, and the oviposition rate of the females were also investigated. Statistical analyses were performed using Student's *t* test.

Protective assay of rIGBP/GST

Three rabbits were inoculated three times with purified rIGBP/GST, as described previously (Gong et al. 2008). Ten days after the last injection, 50 pairs of ticks were fed on each ear of the treated rabbits, and the parameters mentioned in the section above were evaluated. The same dose of GST was used for the negative control.

Results

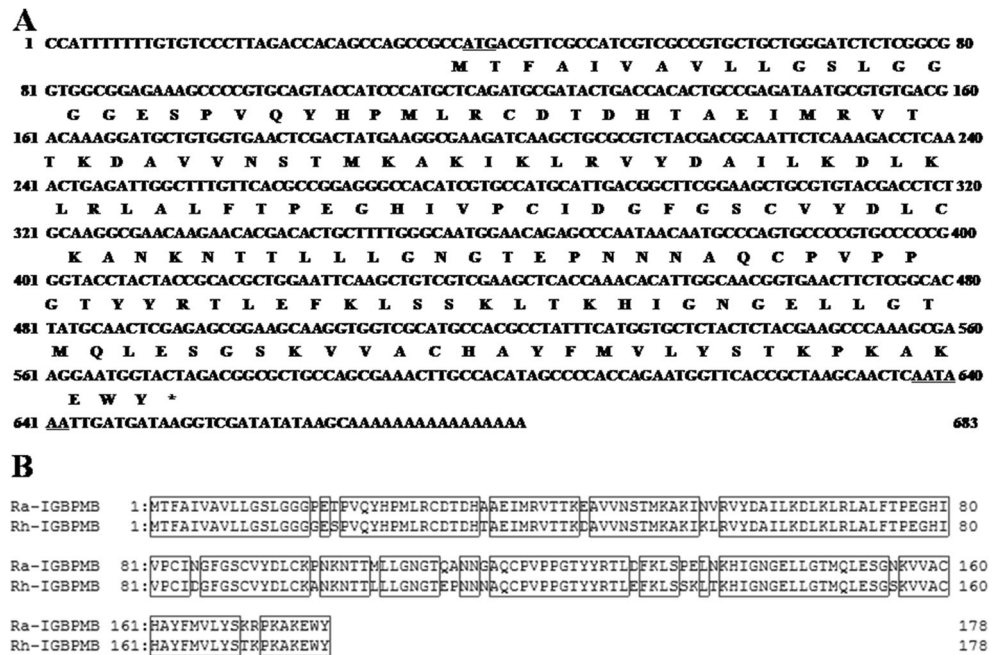
Analysis of the *IGBP* sequence

The full length of *IGBP* was obtained with 3'RACE and 5' RACE and submitted to GenBank (Accession no: DQ115981). The full cDNA consists of 683 bp, with an open reading frame (ORF) of 534 bp, extending from nucleotides 38 to 571, which encodes 178 amino acid residues. The cDNA sequence contains a polyadenylation signal, AATAAA, located 25 bp upstream from the poly(A) tail (Fig. 1a). The deduced protein sequence of IGBP shows a predicted signal peptide of 17 amino acids (SignalP4.1), and the molecular mass of the mature protein is 19.5 kDa. The mature protein sequence of IGBP shares 89 % homology with IGBP B of *R. appendiculatus* (Ra-IGBPMB, AAB68802; Fig. 1b), whereas it shares only 48 % homology with IGBP C of *R. appendiculatus* (Ra-IGBPMC, AAB68803). No obvious domain was detected in the protein sequence with the SMART software.

Expression of the recombinant protein

rIGBP/GST was successfully expressed in *E. coli* BL21(DE3) and was purified to prepare anti-rIGBP/GST antiserum and to investigate its protective effect (Fig. 2a). The *IGBP* sequence was also introduced into the pFastBac HTa vector and expressed as a His-tagged protein in Sf9 cells. As expected, the expressed rIGBP/His was detected at a molecular size of ~23 kDa with both anti-His and anti-rIGBP/GST antibodies (Fig. 2b). A similar band was also detected in the native salivary glands of male ticks (Fig. 2c).

Fig. 1 Analysis of the *IGBP* gene from *R. haemaphysaloides*. **a** Full-length *IGBP* and predicted protein sequence of IGBP. The start codon (ATG) and AATAAA are *underlined*. **b** Alignment of the deduced protein sequence of IGBP from *R. haemaphysaloides* tick (Rh-IGBPMB) with IGBP from *R. appendiculatus* (Ra-IGBPMB, AAB68802)



Expression of IGBP in different developmental stages and tissues of *R. haemaphysaloides* ticks

RNA from the eggs, unfed larvae, unfed nymphs, and unfed adult ticks (females and males) was subjected to an RT-PCR analysis, and as shown in Fig. 3a, *IGBP* was expressed in all stages of the tick. However, it was predominantly found in adult male ticks (Fig. 3b). The highest expression of *IGBP* was in the salivary gland, which was confirmed by both real-time PCR (Fig. 3b) and immunoblotting assays (Fig. 3c). The expression of IGBP in the salivary glands of male ticks was about ten times that observed in female ticks ($P < 0.01$ using Student's *t* test; Fig. 3b), and the IGBP protein product in all the female tick tissues tested was too weak to be detected with the anti-rIGBP/GST antiserum (Fig. 3c). Interestingly, IGBP mRNA was detected, but no protein product appeared in the midguts of male ticks (Fig. 3b and c).

Binding activity of rIGBP/His to different IgGs

Purified IgGs from six kinds of hosts were reacted with rIGBP/His in an ELISA. rIGBP/His bound to the IgGs of rabbit, pig, and dog to different levels. The highest binding was to rabbit and pig IgGs (Fig. 4a). To investigate the binding site on the IgG molecule of the host in detail, the Fc and F(ab')₂ fragments were reacted individually with rIGBP/His. rIGBP/His clearly bound to the F(ab')₂ fragment but not to the Fc fragment (Fig. 4b).

Dynamic changes in the host IgG and IGBP in different tissues of adult ticks

Because IGBP showed the highest binding activity to rabbit IgG, we fed ticks on a BSA-immunized rabbit to investigate the fate of the host IgG. In previous work, Franta et al. (2010)

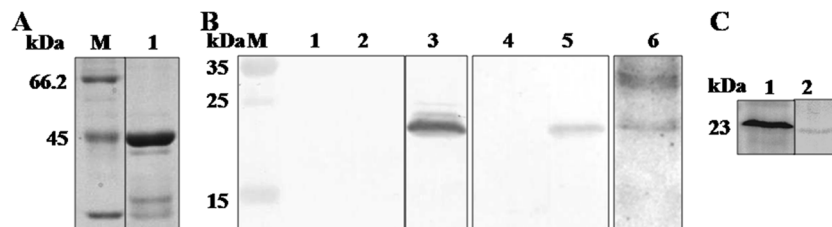
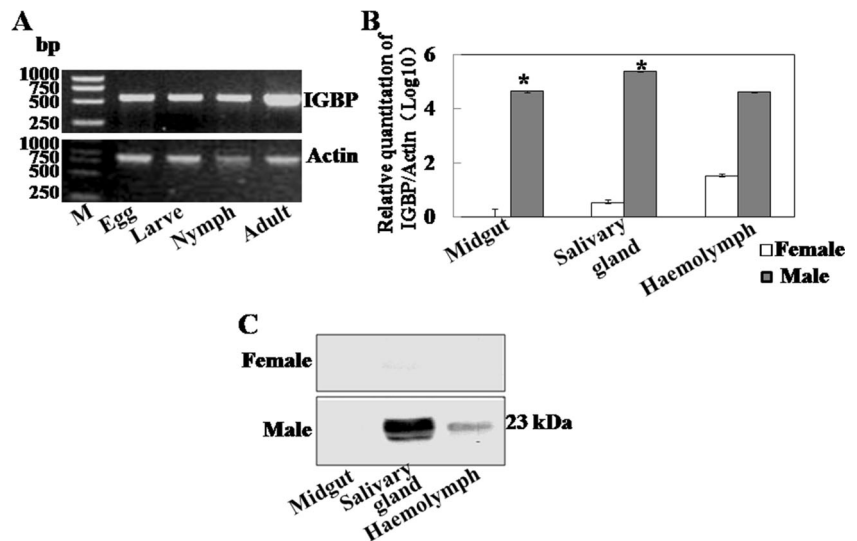


Fig. 2 Expression of the rIGBP protein. **a** Coomassie Brilliant Blue (CBB) staining of rIGBP/GST expressed in *E. coli*. *M*, prestained protein ladder; *lane 1*, purified rIGBP/GST protein. **b** Immunoblot assay and CBB staining of rIGBP/His expressed in Sf9 cells. *Lane 1*, Sf9 cells; *lanes 2 and 4*, Sf9 cells infected with bacmid; *lanes 3 and 5*, Sf9 cells infected with the rIGBP/His bacmid; *lane 6*, purified rIGBP/His (CBB

stained). *Lanes 1–3*, an anti-His monoclonal antibody was used as the primary antibody; *lanes 4 and 5*, anti-rIGBP/GST antiserum was used as the primary antibody. **c** Detection of native IGBP in the salivary glands of male ticks. *Lane 1*, rIGBP/His; *lane 2*, salivary-gland protein from male ticks. Anti-rIGBP/GST antiserum prepared in mice was used as the primary antibody

Fig. 3 Expression analysis of *IGBP* in different tick developmental stages **a** with RT-PCR, and in different tissues from 4-day-fed adult ticks **b** with real-time PCR. **c** Immunoblotting analysis of *IGBP* in different tissues of 4-day-fed adult ticks. Anti-r*IGBP*/GST antiserum was used as the primary antibody. * $P < 0.01$ using Student's *t* test



divided the blood-feeding process of *Ixodes ricinus* into four stages: attachment (day 0–1), slow feeding phase (days 2–6), rapid engorgement phase (days 7–7.5), and detachment (days ~7.5–8). In female ticks of *R. haemaphysaloides*, we found that from the attachment period (day 1), the concentration of host antibodies increased rapidly in all three tissues (including the salivary gland, midgut, and hemolymph), with the greatest increase in the midgut. The concentration of rabbit IgG in the midgut remained stable on days 2–3 or even showed a certain decline 3 days after attachment, followed by a period of rapid increase to the maximum on day 5, where it remained stable until the tick was replete. The IgG levels began to increase on day 4 in the salivary gland and on day 5 in the hemolymph (Fig. 5a). The IgG in the male ticks fluctuated during the feeding progress, and the concentrations of IgG in all three tissues tested decreased to the lowest level on day 4 after attachment. IgG in the salivary gland remained at a very low level (Fig. 5b). At this time, *IGBP* in male ticks appeared from day 4 to the end of engorgement (Fig. 5c), whereas it was only detected in the hemolymph on day 4 after attachment (Fig. 5d).

Effects of *IGBP* interference in adult male ticks

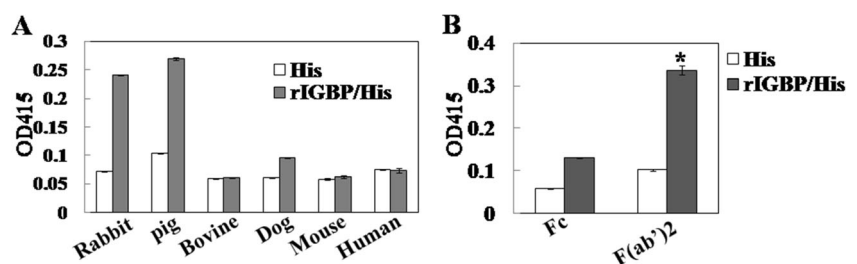
The synthesized dsRNA of *IGBP* and luciferase were injected into adult male ticks, and then the knockdown of *IGBP*

expression was investigated with real-time PCR. As shown in Fig. 6a, *IGBP* was almost silenced in the *IGBP*-dsRNA-injected group, whereas the control group injected with *Luc*-dsRNA demonstrated no reduction. The protein product of *IGBP* was also significantly reduced and could not be detected with anti-r*IGBP*/GST antiserum (Fig. 6b). However, the silencing of *IGBP* expression in the male ticks affected neither the blood-feeding of the female ticks, the subsequent oviposition by the engorged ticks, nor the hatchability of the eggs (Table 2).

Protective activity of r*IGBP*/GST

r*IGBP*/GST was used to immunized a rabbit three times, and then 50 pairs of adult ticks were fed on the rabbit. As shown in Table 3, the bodyweight of the engorged female ticks in the test group (218.9 ± 20 mg) was significantly lower than that of the control group (142.5 ± 43.3 mg; $P < 0.05$ using Student's *t* test), even though their bloodsucking was slightly faster than that of the GST-injected ticks. The mortality of the r*IGBP*/GST-fed ticks was slightly higher than that of the control ticks. However, there was no significant difference in any other parameter between the two groups.

Fig. 4 Binding assay of r*IGBP*/His with IgGs from different hosts with ELISA. **a** Binding assay of r*IGBP*/His with IgGs from different hosts. **b** Binding assay of r*IGBP*/His with different fragments of rabbit IgG. * $P < 0.001$ using Student's *t* test



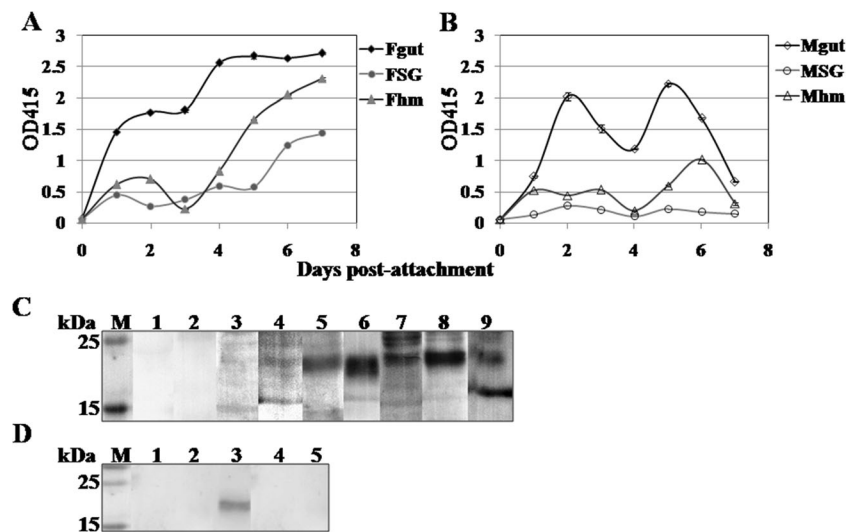


Fig. 5 Dynamic changes in host IgG and IGBP in different tissues of adult ticks. Investigation of rabbit IgG in the tissues of female (a) and male (b) adult ticks 0–7 days after attachment with an ELISA. *Fgut*, female midgut; *FSG*, female salivary gland; *Fhm*, female hemolymph; *Mgut*, male midgut; *MSG*, male salivary gland; *Mhm*, male hemolymph. c

Detection of IGBP in the salivary glands of 0–8-day-fed male ticks. *Lane M*, prestained protein ladder; *lanes 1–9*, salivary gland protein from each day of 0–8-day-fed male ticks. **d** Detection of IGBP in the hemolymph of 0–8-day-fed male ticks. *Lane M*, prestained protein ladder; *lanes 1–5*, salivary gland protein from 0-, 2-, 4-, 6-, and 8-day-fed male ticks

Discussion

Previously, Wang and Nuttall (1995b) detected IGBP-MA, IGBP-MB, and IGBP-MC in male ticks of *R. appendiculatus*. In the present study, we isolated an IGBP from the tick *R. haemaphysaloides*, which showed high homology to IGBP-MB of *R. appendiculatus* but differed greatly from IGBP-MA and IGBP-MC (data not shown). In the tick species *R. appendiculatus*, *A. variegatum*, and *I. hexagonus*, IGBPs have been detected in both the salivary glands and hemolymph of unfed and partially fed female ticks, as well as in male ticks, although different subtypes of the protein have been observed, some even differing in size (Wang and Nuttall 1995a). In the present study, IGBP was detected in all the developmental stages of unfed *R. haemaphysaloides* ticks. However, when we investigated the IGBP in the different tissues of adult ticks, the expression of IGBP was significantly higher in partially fed males than in female ticks. This may suggest that, during the feeding period, the females obtain help from the male ticks to evade damage by host antibodies and supports the opinion that the male tick adopts a role in

“mate guarding” (Wang et al. 1998). Interestingly, IGBP mRNA was highly expressed in the midgut, but the protein product was not detected with immunoblotting (Fig. 3b and c). Because IGBP is a secreted protein according to its signal peptide, this raises the question of where the protein goes after its synthesis. It is well known that the commercial BM86 vaccine can induce antibodies that bind to special epitopes on the midgut and cause the lysis of the gut wall, interfering with the digestion of blood and subsequent egg production (Nuttall et al. 2006). This suggests that IGBPs may not be secreted into the midgut lumen. If this is not the case, the anti-BM86 antibody would bind to IGBP in the lumen instead of on the midgut cell surface, and the antibody would lose its anti-tick effect. However, the absence of IGBP protein in the gut is so far unexplained and requires further investigation.

rIGBP/His showed high binding activity for the IgGs of rabbit and pig but not for the IgGs of cow, mouse, and human, which suggests binding preferences of the protein. The specificity of IGBPs has been widely reported. The results of Wang and Nuttall (1995b) indicated that IGBP-MC from *R. appendiculatus* bound to guinea pig IgG, as well as to

Fig. 6 Confirmation of RNA silencing of IGBP expression in fed male ticks with real-time PCR (a) and immunoblotting assay (b). β -Actin was used as the internal control. *Luci*, luciferase interference; *IGBPi*, IGBP interference. * $P < 0.001$ using Student's *t* test

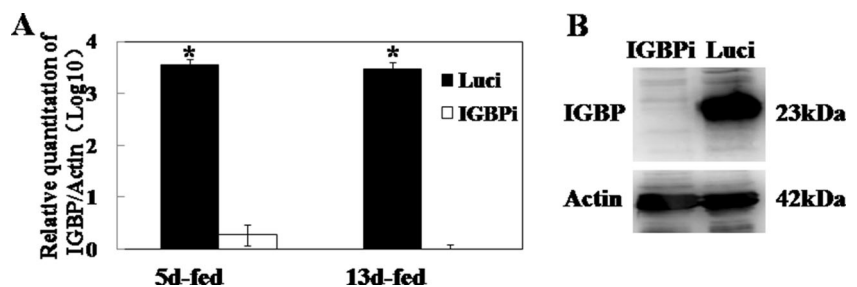


Table 2 Effects of IGBP RNAi on the feeding, oviposition, and hatching behavior of adult ticks

	Luci	IGBPi
Attachment rate of male ticks 24 h after inoculation (%)	68.89	67.74
Survival rate of male ticks 48 h after inoculation	77.78	60.22
Engorgement rate of female ticks (%)	58.59	53.91
Engorged body weight (mg) ^a	222.70±126.81	254.16±141.40
Average engorged time (days) ^a	8.85±0.86	8.69±0.84
Oviposition rate (%)	83.72	83.33
Hatchability (%)	90.28	90.00

^a Average±SD

human and bovine IgG, whereas IGBP-MA and possibly IGBP-MB only bound to guinea pig IgG (Wang and Nuttall 1995b). They also subsequently found that IGBP-MA bound to IgE (Wang and Nuttall 2007), and this activity was used to treat type I hypersensitivity (Wang and Nuttall 2013). This may suggest that different kinds of IGBPs have evolved in ticks to cope with different host responses and to facilitate blood feeding.

To determine the binding site of IGBP, we used an ELISA to check the adhesive activity of rIGBP/His to the Fc and F(ab)₂ fragments of rabbit IgG and found that it clearly bound to the F(ab)₂ but not to the Fc fragment. Carvalho et al. (2011) found an association between the genotype of the constant region of the IgG2 heavy chain and the phenotypes of tick infestations. Furthermore, IgG2 from tick-resistant (*Bos taurus indicus*, Nelore breed) and tick-susceptible (*Bos. t. taurus*, Holstein breed) cattle differ in their hinge regions (Carvalho et al. 2011). Because the hinge region is located in the F(ab)₂ fragment of IgG and IGBP binds to the F(ab)₂ fragment, as demonstrated in the present study, we can infer that IGBP may bind to the hinge region. As we know that ticks ingest large amounts of antibodies during blood meals, the binding of IGBPs to IgGs is considered to be a strategy by which the tick evades the damage caused by the host antibodies. The difference in the ability of hinge regions to bind to IGBP may decide the susceptibility of different cattle breeds

Table 3 Protective effects of anti-rIGBP/GST antiserum on the feeding and survival of adult female ticks

	GST	rIGBP/GST
Average engorged time (day)	10.7±0.2	9.7±1.0
Engorgement rate of female ticks (%)	77.8±9.6	64.5±8.6
Engorgement body weight (mg) ^a	218.9±20	142.5±43.3
Mortality (%) ^a	10.7±5.1	20.6±4.3

^a Student's *t* test, *P*<0.05

to ticks. However, further work is required to test this hypothesis.

The IGBP in male ticks was monitored with immunoblotting during the whole feeding period of the female ticks and was detected in the late stage of the slow feeding phase. This is consistent with a previous description of the abundant production of male-specific IGBPs by *R. appendiculatus* during the late stages of feeding (Wang and Nuttall 1995b). In the late stage of the slow feeding phase of female ticks, the concentration of IgGs in the midgut tends to remain stable, whereas it increases rapidly in the salivary glands and hemolymph. This suggests that the enrichment of IGBPs facilitates the transfer of IgGs and even the return of the IgGs to the host. The abundant expression of IGBPs at this stage may be in preparation for the fast feeding stage in female ticks.

Silencing the expression of IGBP in ticks had no significant impact on their feeding or subsequent oviposition, although the interference of gene expression was confirmed with real-time PCR and immunoblotting. This may be explained by functional compensation by other IGBP molecules. Wang and Nuttall (1995b) identified five kinds of IGBP, ranging from 21 to 54 kDa, in unfed male *R. appendiculatus* ticks. The functions of these proteins probably compensate for one another, although the proteins have highly diverse mRNA sequences. When one kind of *IGBP* gene was silenced, other IGBPs can still be expressed and bind to the host IgGs. However, more data are required to support this hypothesis.

Feeding by female *R. appendiculatus* ticks in the presence of males is partly attributed to an IGBP secreted by the male into the co-feeding site. This phenomenon has been explained as a novel form of “mate guarding,” in which the male helps its mated female evade the immune responses of the host (Wang et al. 1998). Besides co-feeding females, the feeding of larvae and nymphs of *Rhipicephalus sanguineus* and *Amblyomma americanum* is enhanced in the presence of male ticks, which is considered further evidence of the introduction of immunomodulatory saliva proteins, including IGBPs, into the co-feeding site (Rechav and Nuttall 2000). However, if the secreted IGBPs (e.g., IGBP-MA, IGBP-MB, and IGBP-MC) in the feeding site are neutralized by anti-rIGBP/GST antiserum and the expression of IGBP in the female tick itself is as low as indicated in the present study, the feeding process would collapse. To examine this supposition, we investigated the protective activity of rIGBP/GST. As expected, when ticks were fed on rIGBP/GST-immunized rabbits, a reduction of about 35 % in the replete tick bodyweight and mortality rate twice as high as that of the control group were observed (Table 3). Wang et al. (1998) noted that IGBP is a good target for vaccine development. Unfortunately, it seems that one kind of IGBP is ineffective and, based on the results of the present study, cannot be considered a candidate vaccine. However, IGBP may have utility as an adjuvant for vaccination, to facilitate the

antibody–antigen reaction. In summary, this study has provided further important information about tick feeding, as another step towards the development of an anti-tick vaccine.

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