

# Identification of a glycine-rich protein from the tick *Rhipicephalus haemaphysaloides* and evaluation of its vaccine potential against tick feeding

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**Abstract** A cDNA coding a glycine-rich protein was identified from the *Rhipicephalus haemaphysaloides* tick. The cDNA named here as RH50 was 1,823 bp, including a single open reading frame (ORF) of 1,518 nucleotides. The ORF encodes a polypeptide of 506 amino acid residues with a size of 50 kDa, as calculated by a computer. The predicted amino acid sequence of RH50 showed a low homology to sequences of some known extracellular matrix-like proteins. The native protein was identified in both the fed tick salivary gland lysates and extracts of cement material using the serum against the recombinant protein. Reverse transcription polymerase chain reaction results showed that RH50 mRNA was only transcribed in partially fed tick salivary glands, not in unfed tick salivary glands or partially fed tick midgut, fat body, or ovary. The differential expression of RH50 protein in fed tick salivary glands was confirmed by immunofluorescence. The low attachment rate both in the adult and nymphal tick, and the high mortality of immature ticks (nymph) feeding on recombinant RH50-immunized rabbits were found. These results show that the RH50 protein could be a useful candidate for anti-tick vaccine development.

## Introduction

Ticks rank first as arthropod vectors of fungi, protozoa, rickettsiae, bacteria, and viruses, causing diseases in nonhuman vertebrates, and rank only second to mosquitoes as vectors of pathogens to humans (Bior et al. 2002). Tick-borne diseases are particularly devastating to livestock, especially cattle, with costs estimated to be about \$7 billion per annum (Food and Agricultural Organization 1984). Suppression of the tick vector population is considered to control diseases transmitted by ticks (Willadsen and Jongejan 1999) and currently, the principal tick control method is the application of acaricides. However, this method is associated with a number of disadvantages, such as chemical pollution of the food chain and the environment and the rapid development of resistance against acaricides by ticks, thus reducing the effectiveness of acaricides in certain instances (Shapiro et al. 1986). Therefore, it is necessary to develop alternative methods for tick control.

The only currently available commercial vaccine against tick infestation is based on BM86, which is a gut antigen of the host tick *Boophilus microplus* (Riding et al. 1994; Willadsen et al. 1995). However, naturally acquired resistance to ticks is based on a complex combination of host immune responses to a variety of antigens, including a strong cutaneous cell-mediated component against secreted salivary gland peptides (Willadsen 1980). Because resistance to tick feeding was shown to occur utilizing crude salivary gland extracts (Elvin and Kemp 1994), the development of a vaccine that would target tick salivary factors essential for vector–host interactions has now become the focus of tick vaccine development studies (Jaworski 2003; Mulenga et al. 2000). Recently, several vaccine candidate or immunodominant molecules were characterized from tick salivary glands (Mulenga et al. 1999; Tsuda et al. 2001; Bishop et al. 2002).

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The three-host tick *Rhipicephalus haemaphysaloides* is a common ixodid species in China, India, and other south Asian countries (Yin et al. 1997; Miranpuri 1988; Grassman et al. 2004; Dilrukshi et al. 2004). This tick is a major vector of bovine babesiosis in China (Yin et al. 1997) and can also transmit the Kyasanur Forest disease virus, as shown by animal experiments (Bhat et al. 1978). We describe herein the molecular cloning, sequencing, and characterization of a cDNA, designated *R. haemaphysaloides* 50-kDa protein gene (RH50), coding a differentially expressed glycine-rich protein in the salivary glands of *R. haemaphysaloides* ticks and suggest the possible use of the recombinant product for tick control.

## Materials and methods

### Tick and tissue preparation

The *R. haemaphysaloides* ticks were collected from water buffalo in Hubei province in southern China. A single engorged female was used to establish the tick colony; tick rearing was performed in the laboratory in a dark incubator at 25°C with 92% relative humidity by feeding on a New Zealand white rabbit. After three generations had been maintained under laboratory conditions, the colony of *R. haemaphysaloides* was established in Shanghai Institute of Animal Parasitology, Chinese Academy of Agricultural Sciences, Shanghai, China. Dissection of ticks was done as described elsewhere (Mulenga et al. 1999). Briefly, under a dissection light microscope, partially fed ticks were submerged in autoclaved ice-cold phosphate-buffered saline (PBS, pH 7.4) and held down with a pair of soft-tissue forceps. The dorsal cuticle was cut out and the salivary glands were separated from the rest of the organs with the help of 18-gauge needles. After dissection, the salivary glands were pipetted into microcentrifuge tubes, washed once in PBS, and stored at –80°C until use. Dissection of tick organs for RNA extraction was done as described elsewhere (Tsuda et al. 2001). Briefly, unfed or partially fed ticks were rinsed in cold sterile water containing 0.1% diethyl pyrocarbonate (DEPC) and dissection was carried out in the same solution. The separated tick organs were transferred to the RNA extraction reagent, TRIZOL (GIBCO BRL, USA), and stored at –80°C until used for RNA extraction. DEPC was used in the dissection solution to forestall RNase activity.

### Generation of rabbit anti-tick saliva serum

For the generation of polyclonal rabbit anti-tick saliva serum, one rabbit was fed on by ticks at both the immature and mature stages five times. Rabbit anti-tick saliva serum was collected and stored at –80°C.

### Construction and immunoscreening of the tick salivary gland cDNA expression library

Total RNA was prepared from the salivary glands of partially fed female adult ticks, which had remained attached to the rabbit for 4 days. The cDNA library was made using the Clontech SMART cDNA Library Construction Kit following the manufacturer's instructions (Clontech, Palo Alto, CA, USA). The cDNA library ( $10^5$  plaque forming units) was immunoscreened with rabbit anti-tick saliva serum. Immunoscreeing of the cDNA expression library was performed as described previously (You et al. 2001).

### cDNA sequencing and analysis

Using the Dye Terminator cycle sequencing system (Perkin-Elmer, Applied Biosystems, Norwalk, CT, USA) and automated sequencers (Perkin-Elmer and 3100 Genetic Analyzers) the nucleotide sequence of the cloned cDNA was determined by using the vector-specific primers plus gene-specific primers where necessary. Sequence data were analyzed with a computer program (MacVector, version 6.5.3; Oxford Molecular, Hunt Valley, CA, USA).

### 3' and 5' rapid amplification of cDNA ends to clone the full-length gene

Sequence analysis of a positively cloned cDNA revealed that it was a partial gene. To clone full-length cDNAs, we used 3' and 5' rapid amplification of cDNA ends (RACE) according to the manufacturer's instructions (GIBCO). To clone the 5'-end fragments, gene-specific anti-sense primers were designed from DNA sequences of cloned phage cDNAs and used in 5' RACE. After sequencing of the 5'-end fragments, gene-specific sense primers were designed from the extreme N-terminal end and used in 3' RACE to amplify and clone the full-length cDNA. Amplified polymerase chain reaction (PCR) fragments were routinely cloned into a pEGM-T vector, and the nucleotide sequences were determined as described above.

### Expression of recombinant RH50 in *Escherichia coli*

The predicted protein coding the sequence for RH50 in the pEGM-T vector was subcloned into a pET28(a+) *E. coli* expression vector. The resulting plasmid was checked for accurate insertion by sequencing and designated as the pET28/RH50 plasmid. The RH50 gene was expressed in the *E. coli* strain BL21 (DE3) with His-tag according to the manufacturer's instructions (NOVAGEN, USA). For the induction of recombinant RH50 (rRH50) expression, isopropyl-beta-D-thiogalactopyranoside to a final concentration of 1 mM was added and expression was induced for

4 to 6 h at 37°C. The recombinant RH50 protein was purified by Ni affinity chromatography according to the manufacturer's instructions.

#### Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was performed as described previously (Zhou et al. 2002). Cement collection and processing for SDS-PAGE analysis was done as described elsewhere (Bishop et al. 2002). For Western blot analysis, using the prestained protein ladder, extracts of cement material or protein extracts of dissected unfed and 4-day-fed tick salivary glands were electrophoresed on a 12% polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated in the primary antibody (1:200 dilution), and positive signals were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:2,000 dilution) in 3,3'-diaminobenzidine tetrahydrochloride.

#### Expression analysis by reverse transcription polymerase chain reaction

To determine the RH50 gene expression in the salivary glands of unfed and partially fed adult ticks and its expression sites in tick tissues, total RNA extracted from dissected unfed adult tick salivary glands and 4-day-fed adult tick salivary glands, midgut, fat body, and ovary was subjected to reverse transcription (RT)-PCR analysis. To confirm the RH50 gene expression in fed tick of other developmental stage, whole fed larval, nymphal, and adult ticks were used to purify total RNA and then the total RNA was analyzed by RT-PCR. The first-strand cDNA was synthesized from 5 µg of total RNA in a standard RT protocol. One microliter aliquots of the RT products were used in a PCR with gene-specific primers (sense primer, CTTGGAGGTCTTAGTCTTTCTGGTCC; anti-sense primer, TCACACCCTTGCCCCAGTTG). Control RT-PCRs were carried out using tick actin primers to confirm the cDNA integrity (sense primer, GGTTGCCGCCCTGGTGGTTGA; anti-sense primer, GCCGCACGATTCCA TACCCAGG).

#### Indirect fluorescent antibody test

*R. haemaphysaloides* salivary glands were dissected from unfed and 4-day-fed ticks and immobilized on slides. Salivary glands were fixed with methanol containing 2.5% acetone for 20 min. The diluted [1:200 dilutions in 10% fetal calf serum in PBS (FCS-PBS)] anti-rRH50-specific rabbit serum was applied as the first antibody on the fixed

smears and incubated for 30 min at 37°C. After three washings with PBS, Alexa-Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) was subsequently applied (1:500 dilution in FCS-PBS) as a secondary antibody and incubated for another 30 min at 37°C. The slides were washed three times with PBS and then mounted by adding a 50% glycerol-PBS (v/v) solution. The slides were examined under a fluorescence microscope.

#### Immunization and challenge infestation

Six New Zealand white rabbits were used for the immunization and challenge experiment. They were divided into two groups of three. Three rabbits were immunized with the control PBS and the remaining three with rRH50. One milliliter (0.5 mg/ml) of rRH50 or PBS was mixed with the same volume of complete Freund's adjuvant for the initial injection and incomplete Freund's adjuvant for the first and second booster injections, which were given at 2-week intervals. The immunogenicity of rRH50 was analyzed by the reactivity of sera collected from immunized rabbits on Western blots. For challenge infestation, 60 nymphs and 60 adult ticks (30 female ticks) per rabbit ear were introduced and maintained on rabbit ears with the help of ear bags 2 weeks after the booster injection. The ear bags were held onto the rabbit ears with the help of at least three surgical stitches and adhesive tape. To analyze the effects of rRH50-induced anti-tick immunity, a visual examination was conducted 24 h after introducing the ticks on the rabbit ears and then daily from day 3. The parameters analyzed included the attachment rate of 24 h after introduction, period of attachment, mortality, and engorgement weights. Other post-engorgement parameters, such as hatchability and molting rates, were not considered. Mortality of ticks in this study was confirmed by observing all detached and moribund partially fed or engorged ticks at room temperature for about 10 to 20 min. Within this period, all ticks that did not show any mobility was confirmed dead.

#### Nucleotide sequence accession number

The sequence of the RH50 gene of *R. haemaphysaloides* was submitted to the GenBank database under accession number AY550980.

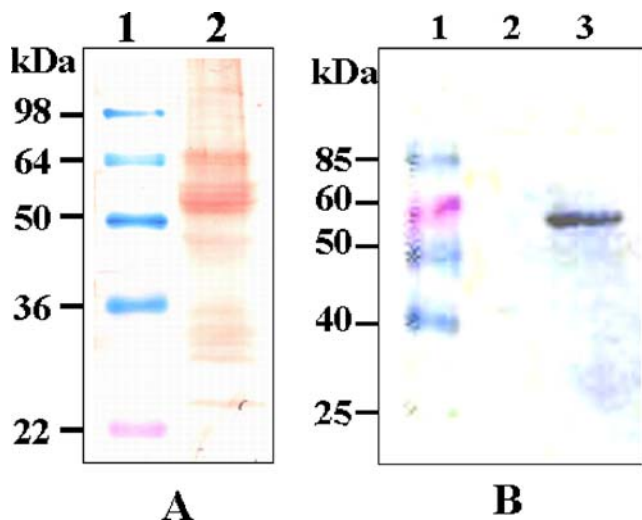
## Results

#### Cloning and sequence analysis of RH50 cDNA

Immunoscreening yielded one phage cDNA, which was 1,616 bp containing poly(A) tail, after sequence analysis showed that it was a partial gene (data not shown). To







**Fig. 2** Western blot analyses of native RH50 protein in the extract of tick cement (**a**) and in the lysate of tick salivary glands (**b**). The samples were electrophoresed on a 12% polyacrylamide gel and blotted onto a PVDF membrane. The membrane was incubated with sera collected from a rRH50-immunized rabbit. **a** Lane 1, prestained marker; and lane 2, the extract of tick cement. **b** Lane 1, prestained marker; lane 2, unfed tick salivary gland lysate; and lane 3, 4-day-fed tick salivary gland lysate

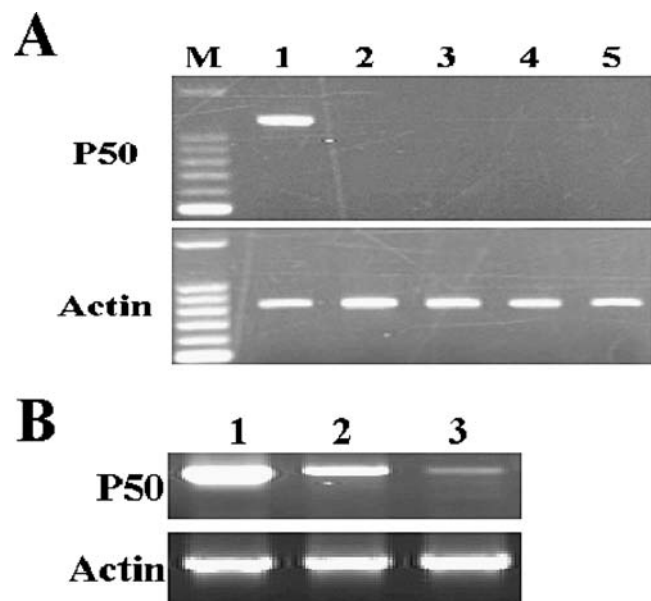
ovary. However, although the control RT-PCR using tick actin-specific primers could detect the special band in all samples, these results showed that RH50 is differentially expressed in salivary glands after blood feeding. Figure 3b shows RH50 also expressed in the fed larval and nymphal ticks.

#### Differentially expressed RH50 protein in tick salivary glands by indirect fluorescent antibody test

The indirect fluorescent antibody test (IFAT) was used to confirm RH50 protein differential expression in tick salivary glands. Figure 4 shows that rabbit anti-rRH50 serum can strongly react with 4-day-fed tick salivary glands, but the unfed tick salivary glands did not show a signal. Control normal rabbit serum did not show a signal in either unfed or 4-day-fed tick salivary glands (data not shown).

#### The vaccination effect of rRH50 against tick feeding in rabbits

The effect of vaccination with rRH50 on tick feeding parameters is summarized in Table 1. For the adult tick, there was a significant low attachment rate ( $P < 0.05$ ) 24 h after introducing ticks on rabbits in vaccination groups (74.7%) compared with the control groups (100%). There was no apparent difference between the vaccination and control groups in feeding period, engorgement body weight, or mortality, although the engorgement body weight was



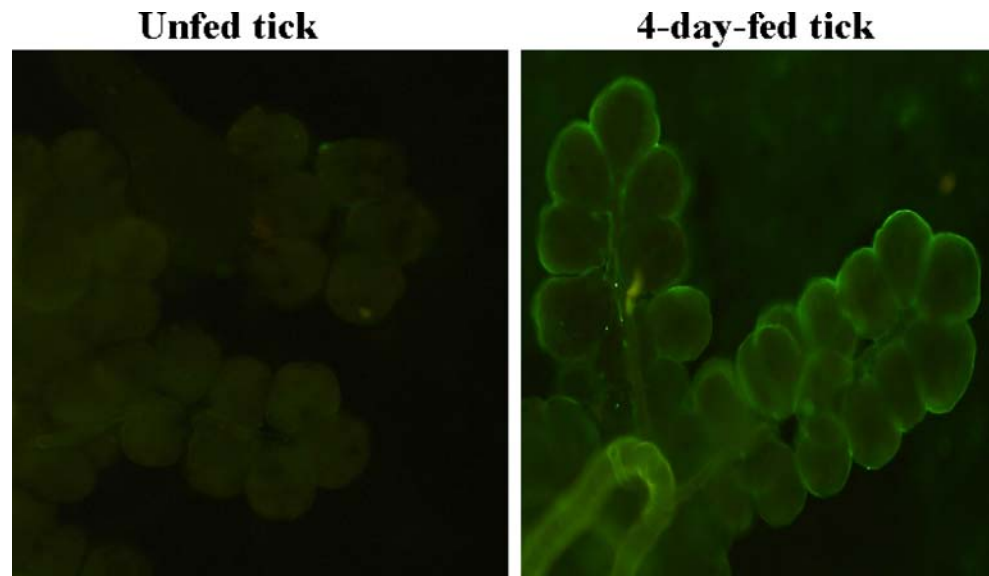
**Fig. 3** Expression analysis of RH50 mRNA. Total RNA extracted from each feeding phase or different tissues were subjected to RT-PCR using specific primers as described in the “Materials and methods.” The predicted product sizes of RH50 and actin genes are about 1.2 and 0.85 kb, respectively. **a** Lane 1, fed adult tick salivary glands; lane 2, unfed adult tick salivary glands; lane 3, fed adult tick midgut; lane 4, fed adult tick fat body; and lane 5, fed adult tick ovary. **b** Lane 1, fed adult larval ticks; lane 2, fed nymphal ticks; and lane 3, fed larval ticks

slightly smaller in the vaccination group. For the nymphal tick, there was also a significant low attachment rate ( $P < 0.05$ ) 24 h after introducing ticks on rabbits in vaccination groups (86.5%) compared with control groups (100%). There was no apparent difference between the vaccination and control groups in feeding duration or engorgement body weight. However, compared with the mortality rate of 13.7% in the control group, the mortality rate in the vaccination group (44.2%) showed a significant increase ( $P < 0.05$ ) of 30.5%. The mortality rate represents the total number of ticks that died on the rabbits and the ticks that died without molting (nymph) or oviposition (adult).

#### Discussion

The current results describe the cloning, DNA sequencing, and characterization of a cDNA coding a glycine-rich protein from the *R. haemaphysaloides* tick salivary glands. We have used a combination of immunoscreening and RACE methods to successfully clone and sequence a full-length cDNA. The cloned cDNA, which we have designated RH50, encoded a protein with a predicted molecular mass of about 50 kDa. The size is consistent with our finding that anti-rRH50 serum recognized an about 56-kDa native protein both in the salivary gland and in the extracts of cement, considering the possible differences between the deduced

**Fig. 4** IFAT for partial fed and unfed tick salivary glands of female ticks with anti-rRH50 serum. The green fluorescent signal was only found in 4-day-fed tick salivary glands



protein and the mature protein. The RH50 protein is rich in glycine, serine, and leucine, which are prominent in the overall amino acid content previously determined for the cement substance of the related ixodid tick *B. microplus* (Kemp et al. 1982). The glycine-rich proteins were found to be expressed in salivary glands of several kinds of hard tick (Mulenga et al. 1999; Bishop et al. 2002; Nene et al. 2004; Untalan et al. 2005; Trimmell et al. 2002). Because of the structural homology of the tick RH50 amino acid sequence to sequences of known extracellular matrix proteins, it is likely to be a membrane-bound matrix, which could be sloughing off from tick salivary gland cells and contaminating in tick saliva. That is the reason why the anti-rRH50 serum can recognize native protein both in the extracts of cement and in the salivary gland lysates. A high glycine content is a feature of vertebrate extracellular matrix

proteins, including keratin and collagen, due to the ability of glycine to adopt a wide range of chain conformations.

Our data provide strong evidence for an upregulation in the expression of RH50 protein in the *R. haemaphysaloides* tick salivary glands after blood feeding. RT-PCR results showed that the RH50 mRNA was only transcribed in partially fed tick salivary glands and not in unfed tick salivary glands or partially fed tick midgut, fat body, or ovary. The differential expression of RH50 protein in fed tick salivary glands was confirmed by Western blot and IFAT using the serum against the recombinant protein. The salivary glands are the largest glands of ticks and in ixodid ticks, they play a major role in feeding (McSwain et al. 1982). It was reported that the overall mass and protein in the salivary glands increase about 25-fold during tick feeding in female *Amblyomma americanum* (Shipley et al. 1993). Specific salivary gland proteins that were shown to change during tick feeding in females include dopamine-activated adenylate cyclase (Schramke et al. 1984), an inhibitor protein of cyclic nucleotide phosphodiesterase (McMullen et al. 1983). Recently, 13 genes differentially expressed in fed tick salivary glands, including a histamine-binding protein, were found in *A. americanum* ticks (Bior et al. 2002). A novel immunosuppressive protein was proven to be differentially expressed in *Ixodes ricinus* salivary glands during the blood meal (Lebouille et al. 2002). The vaccine candidate HL34 and HL35 mRNAs were also induced during feeding (Tsuda et al. 2001). These differentially expressed proteins in tick salivary glands are of major importance to tick survival and modulate innate and acquired immunity. Because specifically expressed salivary gland-associated proteins during tick feeding may play a role in modulating host immune responses and pathogen

**Table 1** Vaccine effect recombinant RH50 on tick feeding

Parameters	Tick stage	Immunized with RH50	Control
Attachment rate (%)	Adult <sup>a</sup>	74.7±4.4* <sup>b</sup>	100±0
	Nymph	86.5±4.7*	100±0
Mortality during and after feeding (%)	Adult <sup>a</sup>	12.3±2.3	10.1±2.4
	Nymph	44.2±3.9*	13.7±1.7
Feeding period (days)	Adult	10.2±1.5	9.8±1.6
	Nymph	5.7±0.6	5.3±0.6
Engorged weight (mg) <sup>c</sup>	Adult	232.2±42.5	248.7±33.4
	Nymph	4.1±0.1	4.3±0.2

\*Statistically significant ( $P < 0.05$ )

<sup>a</sup>Only for female adult ticks

<sup>b</sup>Values were expressed as average±SD

<sup>c</sup>Dead ticks were excluded

transmission, it was suggested to isolate salivary glands of upregulated genes for vaccine development (Xu et al. 2005).

The rRH50 protein expressed in *E. coli* stimulated a specific protective anti-tick immune response in rabbits, as shown by the low attachment rate both in the adult and nymphal tick and the high mortality of immature ticks (nymph) feeding on rRH50-immunized rabbits. Although both the mature and immature ticks commonly express native RH50 protein, as shown by RT-PCR, their sensitivities to the rabbit immune response against RH50 protein appear to be different. Data from studies on p29 and BM86 vaccine tests (Mulenga et al. 1999; Kemp et al. 1986, 1989) provided evidence that may support the hypothesis that immature and mature ticks have different sensitivities to host-acquired resistance against tick molecules.

Vaccination of hosts against ticks was shown to be more practical and sustainable compared to several other tick control strategy (Willadsen 2005). The utilization of tick midgut antigens as effective candidates for induction of anti-tick immunity was documented from the beginning of research area in anti-tick vaccine. Both Bm86 and Bm95, midgut-derived tick antigen, were developed as the vaccine effective against *B. microplus* (Riding et al. 1994; Garcia-Garcia et al. 2000). The idea of using hydrolase and their inhibitors as vaccine candidate was proposed and confirmed by the vaccine effect of recombinant tick serine protease inhibitor (serpin) (Sugino et al. 2003; Imamura et al. 2005). Different from above strategies, tick salivary gland-associated antigens are postulated to be associated with events regulating tick attachment onto the host skin, uptake of the blood meal, and transmission of pathogens, hence, the tick salivary gland is considered as a potential source of tick vaccine antigens (Tsuda et al. 2001). In this study, we provided a new evidence for vaccine effect of salivary gland-associated glycine-rich proteins in another tick species.

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