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Ixodes ticks belonging to the *Ixodes ricinus* complex encode a family of anticomplement proteins

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Abstract

The alternative pathway of complement is an important innate defence against pathogens including ticks. This component of the immune system has selected for pathogens that have evolved countermeasures. Recently, a salivary protein able to inhibit the alternative pathway was cloned from the American tick *Ixodes scapularis* (Valenzuela *et al.*, 2000; *J. Biol. Chem.* 275, 18717–18723). Here, we isolated two different sequences, similar to Isac, from the transcriptome of *I. ricinus* salivary glands. Expression of these sequences revealed that they both encode secreted proteins able to inhibit the complement alternative pathway. These proteins, called *I. ricinus anti*complement (IRAC) protein I and II, are coexpressed constitutively in *I. ricinus* salivary glands and are upregulated during blood feeding. Also, we demonstrated that they are the products of different genes and not of alleles of the same locus. Finally, phylogenetic analyses demonstrate that ticks belonging to the *Ixodes ricinus* complex encode a family of relatively small anticomplement molecules undergoing diversification by positive Darwinian selection.

Keywords: ticks, *Ixodes ricinus*, salivary glands, anticomplement, immune evasion.

Introduction

The complement system is a crucial part of innate immunity. It acts in a cascade manner through two main activation pathways known as classical and alternative (Rother *et al.*, 1998). The classical pathway is activated by the recognition proteins C1q and mannose-binding lectin, which bind, respectively, to charge clusters and neutral sugars on targets. In contrast, activation of the alternative pathway is a default process that proceeds unless downregulated by regulators of complement activation (RCAs). Complement activation results in production of inflammatory anaphylatoxins, deposition of opsonic C3 fragments on surfaces and assembly of the membrane attack complex that disrupts cellular lipid bilayers.

Complement activation on host cells is prevented by several RCAs, whose activities are predominantly restricted to complement of the same species, a phenomenon called homologous restriction (Houle & Hoffmann, 1984; Yamamoto *et al.*, 1990). RCA proteins downregulate complement activity at several steps in the complement cascade (Meri & Jarva, 1998). RCA genes form a gene family, most members of which are clustered in the genome (Rodriguez de Cordoba *et al.*, 1985; Krushkal *et al.*, 2000). RCA proteins share a common structure comprised of several short consensus repeats (SCRs), termed complement control protein repeats (CCPRs), which are functional units. Each SCR contains four cysteines forming disulphide bonds conferring a pearl shape to the SCR domain.

The host complement system exerts a significant evolutionary pressure on pathogens. This component of the immune system has selected for those pathogens, mainly microorganisms but also parasites, that have evolved countermeasures (Cooper, 1991; Blom, 2004). While some pathogens recruit host RCA to their own surface (Thern *et al.*, 1995; Vanderplasschen *et al.*, 1998; Hellwage *et al.*, 2001), others express their own RCA as secreted or membrane associated products (Kotwal & Moss, 1988).

Ixodes ticks are haematophagous ectoparasites responsible for the transmission of important pathogens such as *Borrelia burgdorferi*, *Anaplasma phagocytophila* and *Babesia microti*, the causative agents of Lyme disease, Ehrlichiosis and Babesiosis, respectively. During blood feeding, tick mouthparts remain embedded in the skin of the host for several days (Sonenshine, 1991, 1993). However, only a

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minor inflammatory response is observed when ticks are feeding on their natural hosts. This absence of efficient rejection is due to tick immunomodulating factors secreted into the feeding lesion. A number of host physiological processes, including haemostasis, vasoconstriction, inflammation and pain perception, are targeted by the molecules secreted in tick saliva (Ribeiro *et al.*, 1985; Limo *et al.*, 1991). Several observations suggest that inhibition of the host complement alternative pathway is crucial for the achievement of tick blood feeding. First, it has been demonstrated that the saliva of several *Ixodes* species contains an inhibitor of the alternative pathway (Ribeiro, 1987; Lawrie *et al.*, 2005). Second, the host range of *Ixodes* ticks correlates with their ability to counteract the alternative pathway of their most common host species (Lawrie *et al.*, 1999). Third, cases of host resistance to ticks have been described in which the complement system is implicated with a predominant part of the alternative pathway (Wikel, 1979; Ribeiro, 1987; Lawrie *et al.*, 1999).

A recent study has described the cloning of a novel salivary anticomplement protein from the American tick *I. scapularis* (Valenzuela *et al.*, 2000). This a 18.5 kDa protein, termed *I. scapularis* anticomplement protein or Isac, behaves as a regulator of the alternative pathway in a manner similar to two cellular RCA proteins, decay accelerating factor (DAF) and factor H (Valenzuela *et al.*, 2000). Surprisingly, Isac has no similarity to any previously described anticomplement molecules. However, the presence of four cysteines in Isac suggests that it could be a functional homologue of SCR derived by convergent evolution. Subsequently, several sequences homologous to Isac were described among cDNAs isolated from *I. scapularis* salivary glands (Das *et al.*, 2001; Soares *et al.*, 2005; Ribeiro *et al.*, 2006), and one cDNA homologue of Isac, called ISAC-1, has been reported from *I. pacificus* (Francischetti *et al.*, 2005). The activity of these expression products is still unknown.

I. scapularis and *I. pacificus* belong to the *Ixodes ricinus* species complex, a group of closely related hard ticks comprising at least 14 species distributed throughout the world (Keirans *et al.*, 1999; Xu *et al.*, 2003). The initial goal of the present study was to clone the orthologue of Isac from the European tick *I. ricinus*. Surprisingly, two different sequences homologous to Isac were isolated from the transcriptome of *I. ricinus* salivary glands. Expression of these cDNAs revealed that they both encode functional homologues of Isac, and so we have named these proteins *I. ricinus* anticomplement (IRAC) protein I and II. *In vivo* analyses revealed that IRAC I and IRAC II are encoded by distinct genes rather than alleles of the same locus, and that both IRAC I and IRAC II are coexpressed constitutively in *I. ricinus* salivary glands but are upregulated during blood feeding. Phylogenetic analyses of the *I. scapularis*, *I. pacificus* and *I. ricinus* Isac homologues revealed that ticks belonging to the *I. ricinus* complex encode a family of relatively small anticomplement molecules that have diversified through a process of positive Darwinian selection. The potential of this family of proteins as antigens for the development of an antitick vaccine able to induce an early rejection of the tick and/or inhibition of transmission of tick-borne diseases will be discussed.

Results

Identification of Isac homologues in Ixodes ricinus *ticks*

With the aim of cloning the orthologue of *I. scapularis* Isac from the European tick *I. ricinus*, a reverse transcriptase–polymerase chain reaction (RT–PCR) approach was performed on mRNA purified from salivary glands of engorged adult female ticks. Surprisingly, use of a unique sense primer based on the sequence upstream of the *I. scapularis* Isac open reading frame (Valenzuela *et al.*, 2000) and a poly dT reverse primer, resulted in the amplification of two distinct sequences homologous to Isac. The potential open reading frames within these sequences were called IRAC I and II. Comparison of IRAC I and Isac revealed that they encode 184 amino acids proteins with only 65.8% identity (Fig. 1 and Table 1). The expression product of IRAC II is six amino acids shorter, due to two small deletions not

Table 1.

	Percentage of amino acid or nucleotide identity*											
	IRAC I	Isac	clone 122	IS-18-24-clu513	clones 113/120	Salp20	ISNU-L-ISNU-51	ISNU-L-ISN-50	ISNU-cluster-52	IRAC II	ISAC-1	Salp9
IRAC I		65.8	66.3	66.1	66.9	67.2	66.7	64.9	65.3	66.3	66.8	58.2
Isac	79.8		89.7	90.7	84.3	83.1	86.9	83.3	82.7	68.0	66.8	59.5
clone 122	80.3	95.1		86.8	87.4	87.4	87.4	86.1	84.8	65.7	68.1	59.5
IS-18-24-clu513	80.3	95.3	93.0		87.8	88.5	89.6	86.7	85.0	69.5	67.8	56.4
clones 113/120	80.9	92.5	93.7	94.2		91.9	87.8	85.8	82.1	67.5	68.7	60.6
Salp20	81.2	92.4	94.3	94.9	95.9		91.3	87.3	84.4	69.5	70.1	57.7
ISNU-L-ISNU-51	80.6	94.0	93.7	95.7	93.8	96.2		85.6	85.0	67.8	68.9	61.5
ISNU-L-ISN- 50	80.6	92.6	93.8	93.9	93.1	93.7	93.3		91.9	69.4	68.8	59.5
ISNU-cluster-52	80.3	91.9	93.2	92.3	90.8	92.2	92.3	96.7		68.6	67.4	57.5
IRAC II	82.9	80.3	79.4	80.7	79.4	81.3	80.7	81.4	80.7		80.3	70.9
ISAC-1	83.4	80.4	80.6	80.3	80.4	81.8	81.5	81.4	80.3	91.6		67.1
Salp9	73.3	74.2	74.2	72.2	73.2	73.0	74.7	75.6	73.4	84.6	84.2	

Nucleotide (below diagonal) / Amino Acid (above diagonal)

*Values above and below the diagonal refer to percentage of amino acid and nucleotide sequence identity, respectively.

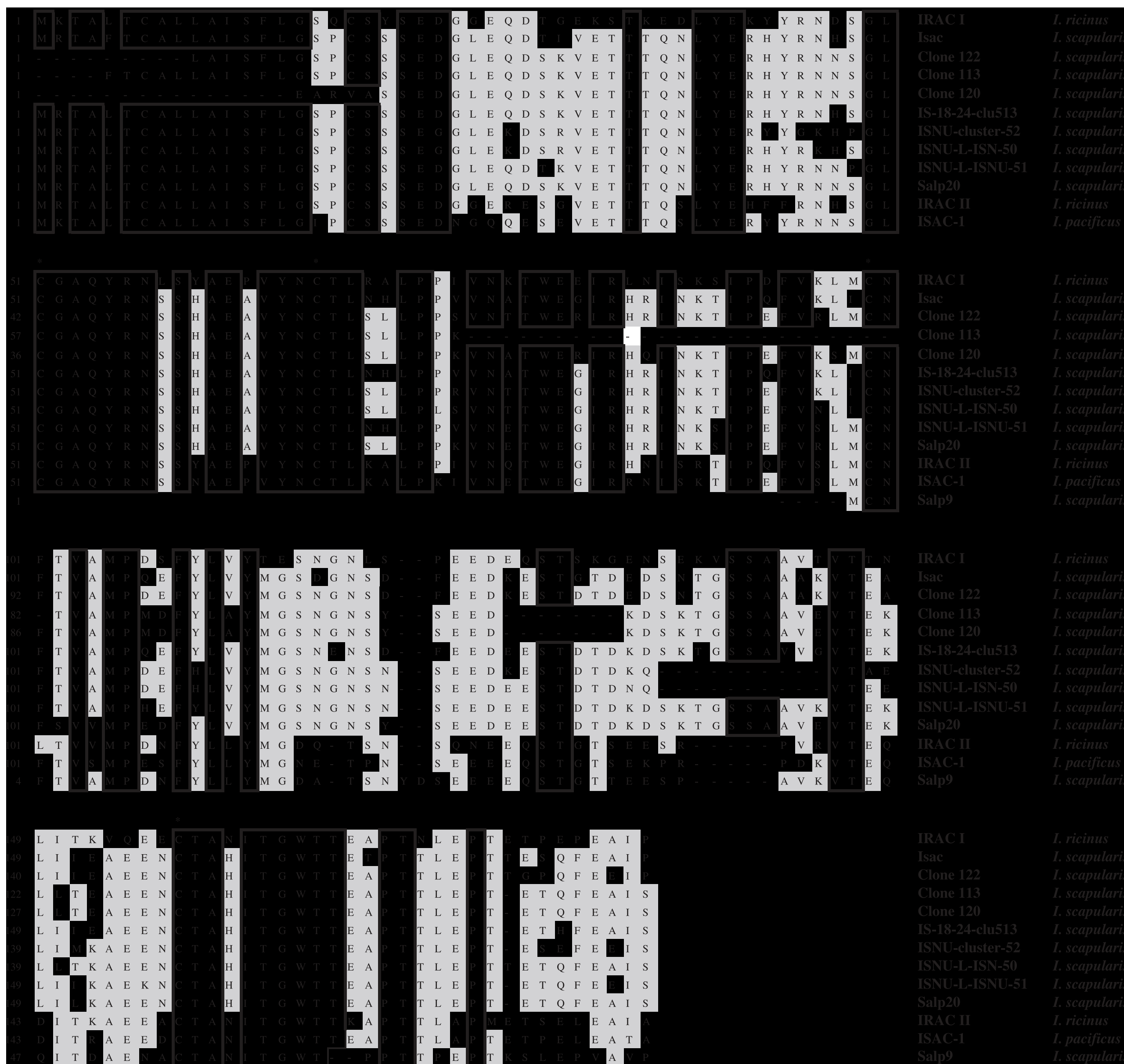

Figure 1. Alignment of *Ixodes ricinus* IRAC I and IRAC II proteins and homologous proteins described from *I. scapularis* and *I. pacificus.* Amino acid residues are numbered with respect to the translation initiation methionine. Residues identical across all sequences are indicated by boxes; those shared by some of the sequences are highlighted in grey. The four cysteines suspected to be important for Isac anticomplement properties are indicated by asterisks.

affecting the reading frame, and exhibits 66.3% and 68.0% amino acid identity with the IRAC I and Isac proteins, respectively (Table 1). Analysis of IRAC I and II sequences suggested that both proteins have a putative N-terminal signal peptide characteristic of secreted proteins (SignalP, CBS, http://www.cbs.dtu.dk/services/SignalP). The putative secreted forms of IRAC I and IRAC II have predicted molecular masses of 20.3 and 19.6 kDa, respectively (Compute pI/Mw Tool, ExPASy, http://uw.expasy.org/tools/pi_tool.html). Importantly, both IRAC proteins possess the four cysteines described in Isac and suspected to be important for its anticomplement activity (Fig. 1). Together these results suggest that IRAC proteins could be functional homologues of Isac secreted in the saliva of *I. ricinus* ticks.

Anticomplement properties of IRAC proteins

To test the ability of the IRAC I and II expression products to inhibit complement, these proteins were expressed in HeLa cells. Concentrated cell supernatants were prepared from cultures transfected with the empty vector or derived vectors encoding IRAC I or IRAC II. Western blot analysis of concentrated cell supernatants revealed that they contained

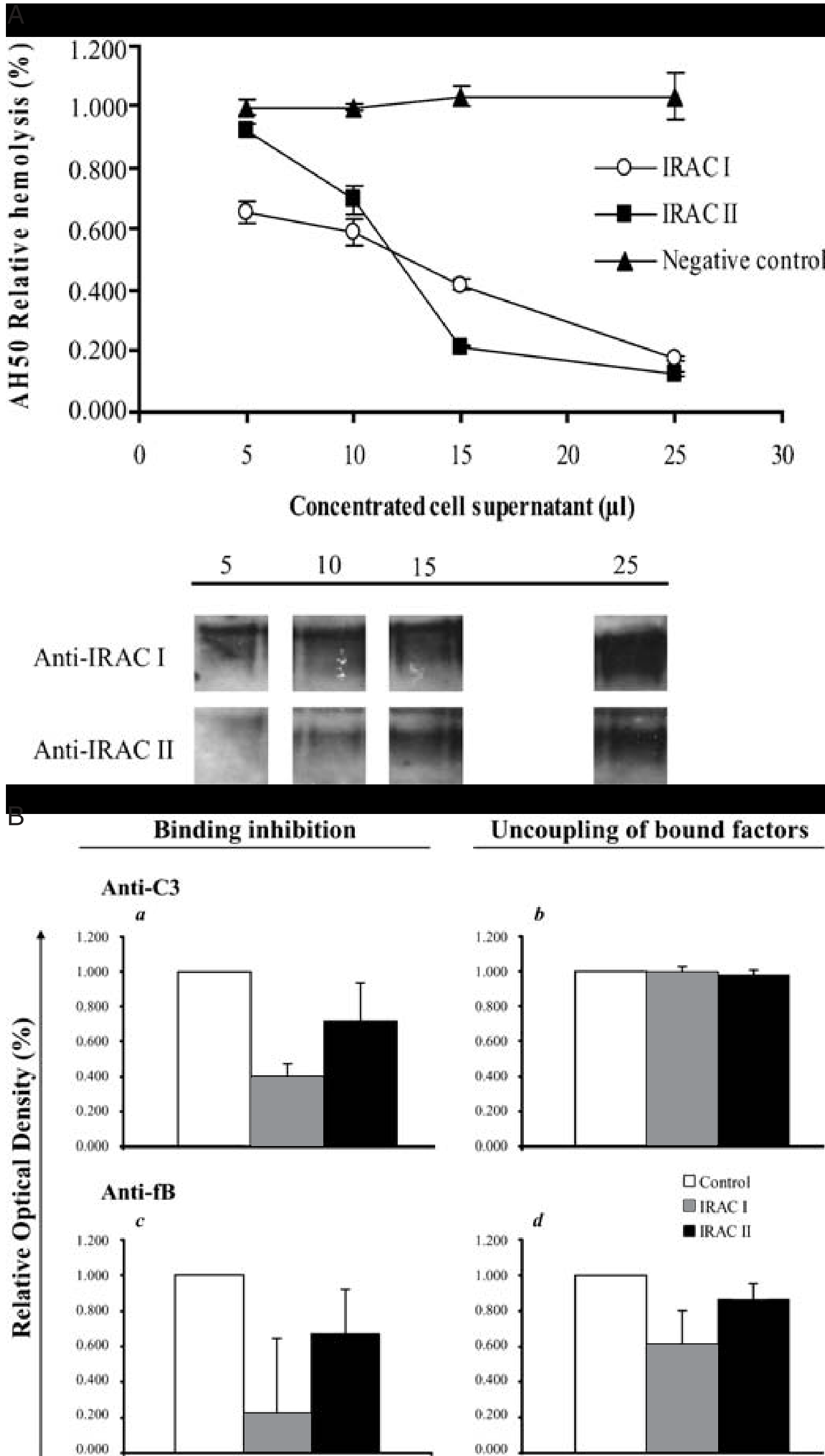

Figure 2. (A) Inhibition of the alternative pathway by IRAC I and IRAC II proteins. HeLa cells were transfected with pcDNA4/TO-IRAC-I, pcDNA4/TO-IRAC-II or pcDNA4/TO vectors and then treated as described in the experimental procedures for production of concentrated cell supernatants. The ability of concentrated cell supernatants to inhibit the complement alternative pathway was assayed by addition of the indicated volume. The data presented are the mean ± SD for triplicate measurements, expressed as the percentage of lysis observed with concentrated cell supernatant from pcDNA4/TO transfected cultures. Bottom of panel A. Immunoblot analysis of concentrated cell supernatants. (B) Effect of IRAC I and IRAC II proteins on C3b and fB deposition to agarose-coated plates. The ability of IRAC I and IRAC II to inhibit the binding of C3b (panel a) or fB (panel c), or to induce the decoupling of prebound C3b (panel b) or fB (panel d) was investigated as described in the procedures. Controls, IRAC I and IRAC II were run with concentrated supernatant from cell cultures transfected with pcDNA4/TO, pcDNA4/TO-IRAC-I and pcDNA4/TO-IRAC-II vectors, respectively. The data presented are the average ± SD for triplicate measurements, expressed as percentages of the optical density observed for controls.

similar amounts of IRAC I or IRAC II proteins. Analysis of these supernatants did not reveal any inhibitory effect of IRAC I or IRAC II on the classical pathway (data not shown). However, a dose-dependent inhibition of the alternative pathway was observed for both IRAC I and IRAC II (Fig. 2A) as measured by their ability to inhibit the lysis of rabbit erythrocytes by human serum. Statistical analysis of IRAC I and IRAC II dose–effect curves revealed that they are significantly different ($P < 0.05$) and suggests that IRAC II is more effective than IRAC I under the conditions of this assay. Taken together, these results demonstrate that IRAC I and IRAC II are secreted inhibitors of the complement alternative pathway.

To further characterize the anticomplement activity of IRAC molecules, concentrated cell supernatants were submitted to C3b and fB ELISA tests as described previously by Valenzuela *et al.* (2000) (Fig. 2B). These assays revealed that IRAC I and IRAC II inhibited the binding of C3b to agarose-coated wells, but did not uncouple covalently prebound C3b (Fig. 2B, panels a and b). Factor B attaches noncovalently to C3b. Both IRAC molecules inhibited the binding of fB to C3b and induced the displacement of prebound fB (Fig. 2B, panels c and d).

Immunodetection of IRAC proteins in tick salivary glands

With the goal in mind to detect IRAC molecules *in situ*, mouse polysera were raised against IRAC molecules expressed as His-tagged proteins. Immunostaining of HeLa cells expressing IRAC I or IRAC II with the polysera obtained revealed a strong staining of expressing cells but also a cross-reaction of the polysera for the two proteins (Fig. 3, compare panels c and d or panels e and f). To avoid the latter problem resulting from the homology existing between IRAC molecules, monoclonal antibodies (mAbs) specific of IRAC I or IRAC II were produced. Their specificity is demonstrated in Fig. 3B. These mAbs were then used to stain cryosections performed through unfed or 4-day engorged ticks. These stainings revealed that both proteins are expressed constitutively in *I. ricinus* salivary glands and are upregulated during blood feeding (Fig. 4). Stainings performed with an isotypic control mAb demonstrated the specificity of the signals observed.

Identification of IRAC I and II as the expression products of different genes

The extent of divergence between the IRAC I and IRAC II sequences (Fig. 1 and Table 1) is much greater than would be expected for alleles from one locus, strongly suggesting that these proteins result from the expression of two different genes. To test this hypothesis, 12 ticks collected from different locations in Belgium were microdissected: for each tick, left and right salivary glands were isolated and stained with mAbs specific to IRAC I and IRAC II, respectively (Fig. 5). All 12 ticks were found to express both IRAC I and IRAC II. Even at the maximum possible expected heterozygosity (i.e. 0.5 for two alleles, when they have equal frequencies), the chance of finding 12 heterozygote ticks (i.e. ticks expressing both IRAC I and IRAC II) among a sample of 12 is extremely small ($P = 0.5^{12} = 0.00024$). Thus, these expression data lend strong support to the hypothesis that IRAC I and IRAC II are encoded by separate genes.

Evolutionary relationships of the IRAC genes

A number of sequences homologous to Isac, IRAC I and IRAC II have been reported. One, ISAC-1, was isolated from *I. pacificus* salivary glands (Francischetti *et al.*, 2005). The others were isolated from *I. scapularis* salivary glands: Salp9 and Salp20 (Das *et al.*, 2001), clones 113, 120 and 122 (Soares *et al.*, 2005), and isolates ISNU-L-ISN-50, ISNU-L-ISNU-51, ISNU-cluster-52 and IS-18-24-clu513

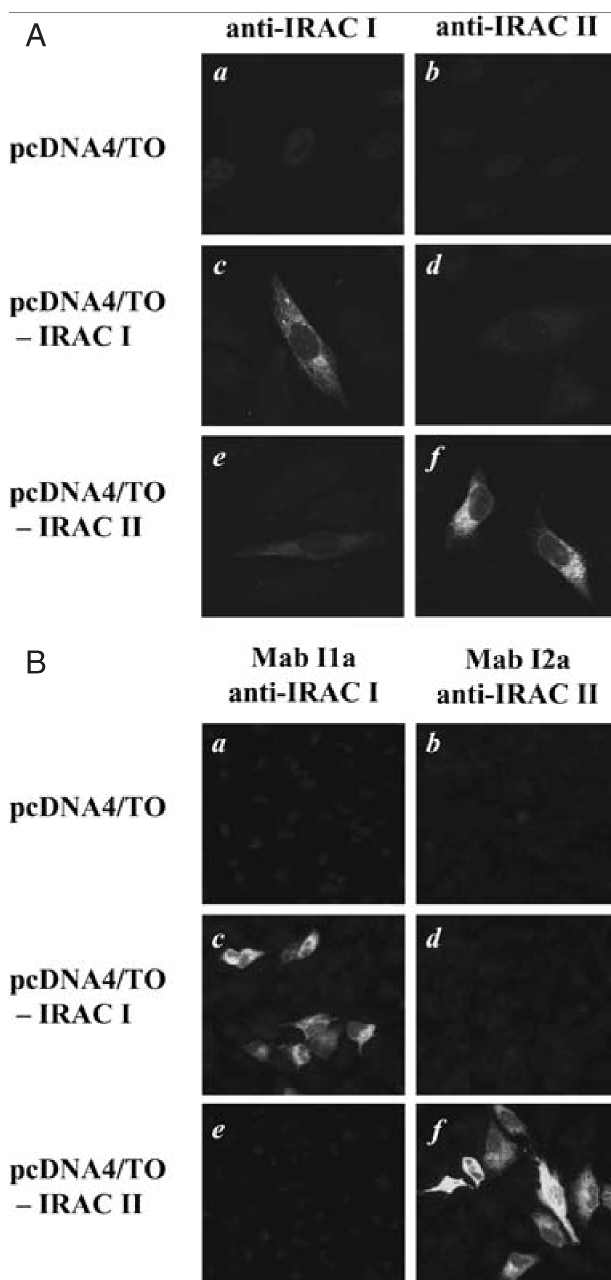

Figure 3. Immunofluorescent detection of IRAC I and IRAC II. HeLa cells were transfected with pcDNA4/TO (panels a and b), pcDNA4/TO-IRAC-I (panels c and d), and pcDNA4/TO-IRAC-II (panels e and f) vectors. Thirty-six hours after transfection, the cells were treated for indirect immunofluorescent staining: mouse serum anti-IRAC I (part A, panels a, c and e), mouse serum anti-IRAC II (part A, panels b, d and f), mAb I1a raised against IRAC I (part B, panels a, c and e) and mAb I2a raised against IRAC II (part B, panels b, d and f) were used as first antibodies and were revealed by Alexa 488-GAM. The side of each panel in parts A and B corresponds to 125 and 250 μm of the specimen, respectively.

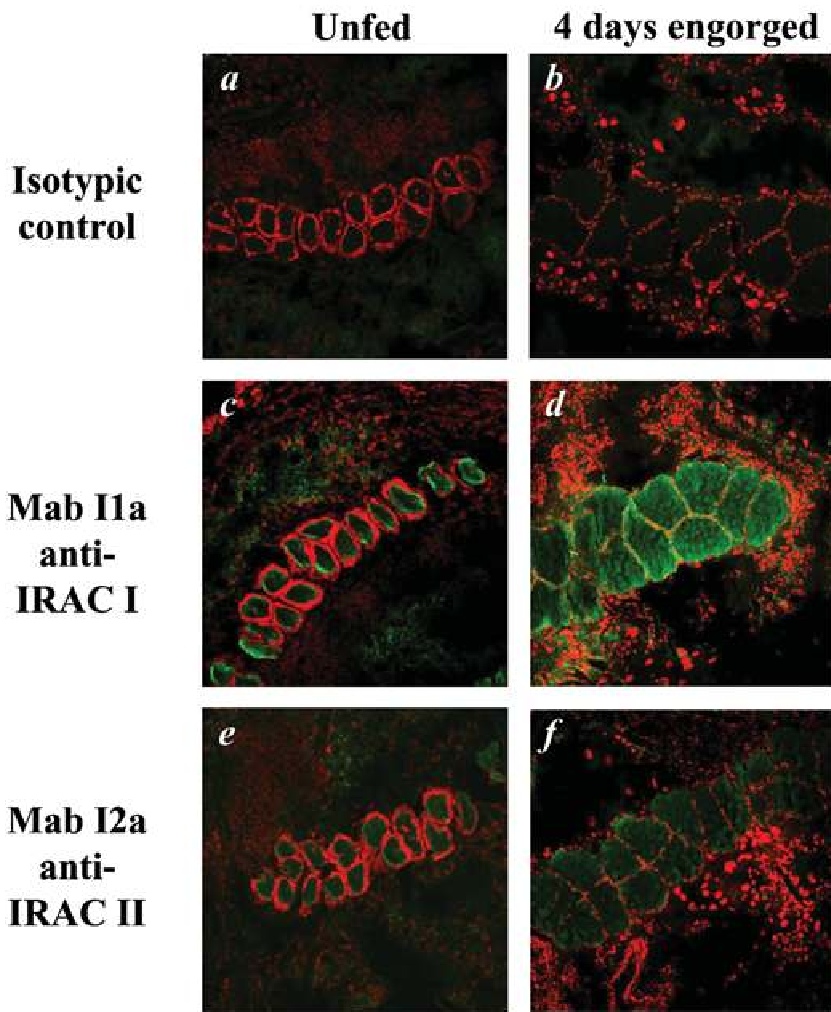

Figure 4. Expression of IRAC I and IRAC II proteins in *Ixodes ricinus* salivary glands. Parafrontal cryo-sections were performed in unfed (panels a, c and e) or 4 days engorged (panels b, d and f) adult female *I. ricinus* ticks. Sections were treated for indirect immunofluorescent staining: mAb 198 (isotypic control), mAb I1a raised against IRAC I (panels c and d) and mAb I2a raised against IRAC II (panels e and f) were used as first antibodies and were revealed by Alexa 488-GAM. Cell nuclei were stained with TO-PRO-3 iodide. Sections were examined by confocal microscopy for green (Alexa-GAM) and red (TO-PRO-3 iodide) fluorescent signals. Pictures represent the merged image of the red and green emissions. The side of each panel corresponds to 1000 µm of the specimen.

(Ribeiro *et al.*, 2006). Among these, clones 113 and 120 differed in length but otherwise had identical sequences, and so were combined as a single sequence.

We investigated the evolution of these genes by phylogenetic analyses of their aligned protein sequences. Owing to the truncated nature of the Salp9 sequence (Fig. 1) analysis of all 12 sequences was restricted to 66 aligned residues (Fig. 6A), whereas analysis of 11 sequences (excluding Salp9) included 156 sites (Fig. 6B). The sequences fall into three clades: one including one sequence from each of the three species (IRAC II, ISAC-1 and Salp9), one including eight sequences from *I. scapularis*, and one comprised of *I. ricinus* IRAC I only. The only topological differences between the two trees concern the position of clone 122 relative to the other *I. scapularis* sequences: over the 3′ region of the gene clone 122 is most closely related to Isac (Fig. 6A), whereas when the whole sequence is considered clone 122 does not seem specifically closely related to any of the other *I. scapularis* sequences (Fig. 6B). This appears to reflect recombination events during the divergence of these sequences.

While the phylogenetic analyses yield unrooted trees, the simplest interpretation would seem to be to place the root between the IRAC II clade and the other sequences (as in Fig. 6); then the root of the tree reflects a gene duplication in an ancestral species. To one side of the root, IRAC II, ISAC-1 and Salp9 seem to represent orthologous genes in *I. ricinus*, *I. pacificus* and *I. scapularis*, respectively. To the other side of the root, there appear to have been multiple gene duplication events within the *I. scapularis* lineage. IRAC I could be the orthologue of this *I. scapularis* gene family, although this would imply a much faster rate of divergence among the IRAC I-like sequences than among the IRAC II-like sequences.

The interpretation of IRAC II, ISAC-1 and Salp9 as being orthologous does not seem consistent with an earlier phylogenetic analysis based on a short segment of the mitochondrial 16S rRNA sequence, which placed *I. ricinus* as an outgroup to *I. scapularis* and *I. pacificus* (Black & Piesman, 1994). While our phylogeny (Fig. 6A) was derived from protein sequences, the greater similarity between *I. ricinus* and *I. pacificus* is also seen at the nucleotide level, in terms of

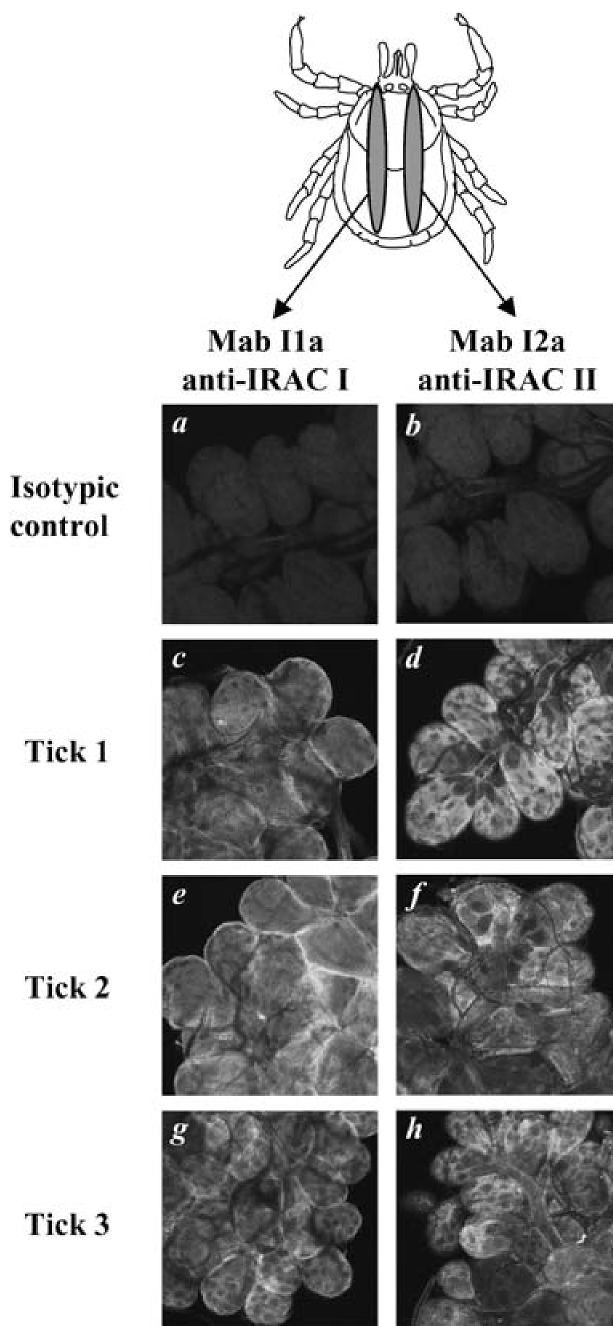

Figure 5. Expression of IRAC I and IRAC II proteins in different *Ixodes ricinus* ticks. Twelve adult engorged female ticks were collected on game animals killed in various forests throughout Belgium. Micro-dissected left and right salivary glands were incubated with mAb I1a raised against IRAC I (panels c, e and g) and mAb I2a raised against IRAC II (panels d, f and h), respectively. Panels a and b represent salivary glands incubated with mAb 198 used as an isotypic control. Primary antibodies were revealed by Alexa 488-GAM. The side of each panel corresponds to 500 μm of the specimen. Representative results obtained from three ticks are presented in the figure; similar results were obtained with all ticks tested.

Table 2. Extent of divergence among *Ixodes* species. Divergence for protein-coding sequences was estimated as the numbers of synonymous (K_S) and nonsynonymous (K_A) substitutions per site, by Li's method (Li, 1993), applied to L codons compared. The average G + C content at synonymously variable third positions of codons (GC3s) is also shown. For IRAC II vs. ISAC-1, a second comparison is made (*) restricted to the region available for Salp9

Gene	K_S	K_A	K_A/K_S	L	GC3s
I. ricinus vs. *I. scapularis*					
IRAC I/Isac	0.277	0.244	0.88	184	0.42
IRAC I/Salp20	0.241	0.224	0.93	183	0.44
IRAC I/ISNU-L-ISN-50	0.276	0.228	0.83	174	0.44
IRAC I/ISNU-cluster-52	0.304	0.221	0.73	173	0.43
IRAC II/Salp9	0.156	0.182	1.17	79	0.51
Calreticulin	0.097	0.006	0.07	413	0.88
Ferritin	0.095	0.008	0.08	172	0.92
Metalloprotease	0.138	0.062	0.45	339	0.50
RP S18	0.050	0.004	0.07	146	0.79
Actin	0.035	0.004	0.11	136	0.89
I. ricinus vs. *I. pacificus*					
IRAC II/ISAC-1	0.047	0.110	2.33	178	0.48
IRAC II/ISAC-1*	0.037	0.134	3.58	81	0.49
Calreticulin	0.053	0.006	0.11	413	0.88
Metalloprotease	0.106	0.052	0.49	339	0.49
I. scapularis vs. *I. pacificus*					
Salp9/ISAC-1	0.130	0.200	1.54	79	0.50
Calreticulin	0.067	0.006	0.09	413	0.88
Metalloprotease	0.131	0.055	0.42	340	0.50

K_S, the extent of divergence at silent sites (Table 2). The sequences of two other protein-coding nuclear genes, encoding calreticulin and a metalloprotease, are available from all three species: in both cases, the smallest interspecies difference is between *I. ricinus* and *I. pacificus* (Table 2), as for IRAC II and ISAC-1. Thus, the nuclear gene sequences consistently indicate a relationship among these three species different from that obtained from mitochondrial sequences.

The extent of synonymous nucleotide substitution among IRAC II, ISAC-1 and Salp9 is consistent with these genes being orthologues (Table 2). For example, excluding the anticomplement protein genes, K_S values for genes available for comparison between *I. ricinus* and *I. scapularis* range from 0.035 to 0.138. This variation probably reflects constraints on silent sites due to selection on codon usage, to maximize the efficiency and/or accuracy of translation. Such selection on codon usage has been reported for another arthropod, *Drosophila*, where highly expressed genes have strongly biased, G + C-rich, codon usage (Shields *et al.*, 1988; Duret & Mouchiroud, 1999). In the comparison between *I. ricinus* and *I. scapularis*, the four genes with the most G + C-rich codon usage have the smallest values of K_S, consistent with selective constraint; in contrast, IRAC II/Salp9 and the metalloprotease gene both have relatively unbiased codon usage, and similar (higher) K_S values. Conversely, the K_S values for comparisons between IRAC I and *I. scapularis* homologues are much higher

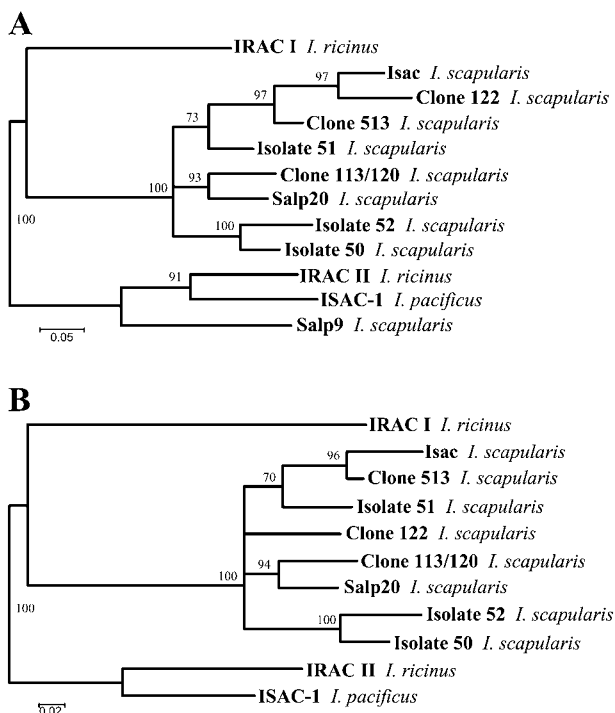

Figure 6. Phylogenetic relationships among the protein sequences of IRAC homologues. (A) Twelve sequences compared across 66 aligned residues. (B) Eleven sequences (Salp9 excluded) compared across 156 aligned residues. Horizontal branch lengths are drawn to scale, with the scale bars representing 0.05 (A) and 0.02 (B) replacements per site. Numbers on internal branches indicate estimated posterior probabilities.

(Table 2), suggesting that they may have been diverging since before the speciation of *I. ricinus* and *I. scapularis*.

Adaptive evolution of IRAC genes

The ratio of nonsynonymous vs. synonymous substitutions (K_A/K_S) can be used to make inferences about the pattern of evolution in genes (Sharp, 1997). This ratio is normally much lower than 1, reflecting the constraint on nonsynonymous substitutions, and the relative lack of constraint on synonymous substitutions; for example, among a large sample of genes compared between mouse and rat, the median value was 0.08 (Sharp, 1997). Values similar to this are seen for the calreticulin, actin, ferritin and RP S18 genes, despite their apparently constrained K_S values, while that for the metalloprotease gene is somewhat higher (Table 2). In contrast, in the comparisons of IRAC II, ISAC-1 and Salp9 the K_A/K_S values are greater than 1. Values of the K_A/K_S ratio can increase either by a relaxation of selective constraint on the protein sequence or by positive selection for amino acid changes, but only the latter force can elevate the values above 1. Furthermore, it is difficult for the ratio to be greater than 1, even with positive selection for amino acid changes, unless there is selection of a large number of changes over a short period of time (Sharp, 1997). Thus, the high values of K_A/K_S seen for IRAC II, ISAC-1 and Salp9 are strong evidence that these sequences have been subject to positive diversifying selection for amino acid changes during their evolution. The values for IRAC I compared with the most closely related *I. scapularis* homologues were also unusually high (Table 2); although these values were a little under 1, they are more likely to reflect positive diversifying selection than a near complete absence of selective constraint.

Discussion

Several distinct arthropod groups have independently evolved haematophagous behaviour. The ancestors of *Ixodes* ticks were scavengers (Mans & Neitz, 2004). To develop their haematophagous behaviour characterized by a long continuous blood meal, *Ixodes* ticks had to acquire mechanisms to counteract several host physiological processes. Several observations listed in the introduction indicate that the alternative pathway of the host immune system has exerted a strong selective pressure on these parasites during their evolution. Reinforcing this interpretation, the present study suggests that ticks belonging to the *I. ricinus* complex have a gene family encoding multiple anticomplement proteins, evolving under positive diversifying selection.

The *Ixodes* family of anticomplement proteins does not appear to have detectable homology with any previously reported sequences. In particular, IRAC homologues do not contain any amino acid motifs found within the SCR of RCA (McLure *et al.*, 2004). However, like SCRs, all IRAC homologues (except the apparently incomplete Salp9 sequence) contain four conserved cysteines essential for the anticomplement activity of RCA. These data suggest that the anticomplement activity of the proteins reported here has been acquired through a process of convergent evolution. *Ixodes* ticks are hard ticks that differ from related soft ticks in several morphological features as well as their feeding behaviour. However, soft ticks have apparently developed their haematophagous behaviour independently of hard ticks (Mans & Neitz, 2004), and so it would be interesting to characterize the anticomplement activity of soft tick saliva, to identify the genes responsible for this activity and study their phylogenetic relationship, if any, with the IRAC homologues. The identification of additional novel salivary anticomplement molecules acquired through convergent evolution by blood feeding arthropods would provide further support for the importance of complement inhibition in the host–parasite relationship. Interestingly, a recent study described the identification of a complement inhibitor of C5 activation from the soft tick *Ornithodoros moubata* (Nunn *et al.*, 2005); this molecule, termed OmCI, is the first lipocalin family member shown to inhibit complement activation.

The data presented in this study revealed that both IRAC I and II exhibit anticomplement properties similar to those described for Isac (Fig. 2). Analysis of the expression of IRAC I and IRAC II in different ticks (Fig. 5), as well as consideration of the extent of divergence between the IRAC I and II sequences, indicate that they are paralogues generated by gene duplication, rather than allelic variants. In phylogenetic analyses Isac is more closely related to IRAC I than IRAC II, but again the extent of divergence between IRAC I and Isac is so great, even at silent sites, as to suggest that Isac and IRAC I are also paralogues (Fig. 6, Table 2). The selective advantage conferred by expressing these paralogous proteins could be explained by a synergistic effect resulting from their coexpression. Alternatively, the different paralogous proteins may have different inhibitory activities against the complement of different host species; it will be interesting to investigate whether the combination of IRAC I and IRAC II has increased the host range of *I. ricinus* ticks. The anticomplement activity of the other homologous sequences has not been tested so far. As described above, it is possible that diversifying selection has led to these sequences acquiring altered biological properties.

Two types of vaccines are possible against ectoparasites such as ticks, using antigens either 'concealed' or 'exposed' to the immune system during natural infestation. A vaccine using 'concealed' antigens is already commercially available (TickGARD™ in West Ride, Australia and Gavac™ in Havana, Cuba). These vaccines relied on the Bm86 antigen, a midgut membrane-bound protein of the *Boophilus microplus* tick (Willadsen *et al.*, 1989). This vaccination induces an antibody-dependent immunity that causes destruction of the midgut and leaking of the ingested blood into the body cavity of the tick inducing death or reducing fertility (Rand *et al.*, 1989). Notwithstanding the ingenious aspect of 'concealed' antigen-based vaccines, they exhibit several disadvantages. First, they confer only short-term protective immunity because natural infestation does not boost the immune system. Consequently, these vaccines require frequent boosters. Second, the immune response induced by 'concealed' antigen based vaccines does not induce an early rejection of the tick and consequently is unable to prevent the transmission of the pathogens carried by the parasite. 'Exposed' antigen-based vaccines should avoid these disadvantages.

Two main arguments suggest that IRAC proteins are good 'exposed' candidate antigens for the development of an antitick vaccine, which could interrupt the tick blood meal shortly after the bite, and consequently reduce the risk of transmission of tick-borne pathogens. First, cases of host resistance to ticks have been described in which the complement system is implicated with a predominant part of the alternative pathway (Wikel, 1979; Ribeiro, 1987; Lawrie *et al.*, 1999); consequently, it is crucial to block tick proteins able to inhibit this part of the immune system. Second, IRAC I and IRAC II were proved to be expressed constitutively in the saliva of unfed ticks (Fig. 4) suggesting their importance in the earlier steps of the blood meal. This constitutive expression ensures that IRAC molecules will be among the first antigens injected into the host skin. Inducing an appropriate humoral immune response could have several advantages for the host. Neutralization of IRAC molecules by antibodies should allow the default activation of the alternative pathway. Moreover, the immune complexes generated by the binding of the host antibodies on IRAC molecules should activate the classical pathway. All together, the activation of these two pathways should generate an inflammation that could induce the interruption of the blood meal. It is also important to note that the anticomplement activity mediated by IRAC homologues represents a mechanism of protection against the host complement for the pathogens transmitted by the tick saliva. Inhibition of IRAC homologues should preserve the alternative pathway of the host at the site of the bite and therefore maintain the ability of the host immune system to control the infection at a very early stage. Despite the homology existing between IRAC proteins, the results presented in Fig. 3 (panel B) suggest that a vaccine based on these molecules should contain epitopes from both paralogues, or even epitopes from each member of the anticomplement protein family. Experiments to test the potential of IRAC molecules as antigens for the development of an antitick vaccine are in progress.

In conclusion, the present study demonstrates that ticks belonging to the *Ixodes ricinus* complex encode a family of anticomplement proteins that represent a group of candidate antigens for the development of an antitick vaccine potentially able to induce an early rejection of the tick and/or inhibition of transmission of tick-borne diseases.

Experimental procedures

Purification of mRNA from 5-day-fed tick salivary glands

Salivary glands of 50 adult female *I. ricinus* ticks from a laboratory colony engorged for 5 days on the ears of New Zealand White rabbits were dissected and immediately frozen in liquid nitrogen. To extract mRNA, salivary glands were crushed in liquid nitrogen using a mortar and a pestle. Purification of approximately 400 ng of mRNA was then performed using the Fast Track 2.0 kit (Invitrogen, Merelbeke, Belgium).

Isolation of cDNA encoding IRACs by reverse transcriptase–polymerase chain reaction

RT reactions were performed on 150 ng of *I. ricinus* salivary gland mRNA using First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, NJ). The 5′-*Not*I-$d(T)_{18}$-3′ primer provided in the kit was used for these reactions. Finally, cDNA products were amplified by PCR using the forward primer 5′-ACAGCACTGAGGTTTCAGA G-3′ and the reverse primer 5′-*Not*I-$d(T)_{18}$-3′. The former primer was designed based on Isac cDNA sequence (nucleotides −36 to −17 according to the translation initiation site, GenBank

accession number AF270496). RT–PCR products were TA-cloned into the pGEM-T easy vector (Promega, Madison, WI) and sequenced.

Nucleotide sequence accession numbers

The sequences reported in this paper have been deposited in the GenBank database: IRAC I cDNA (AY878714) and IRAC II cDNA (AY878715). The following previously published sequences were also used in this study: Isac (AF270496), Salp9 (AF278574), Salp20 (AF209917), clone 113 (AY956386), clone 120 (AY956387), clone 122 (AY956388), isolate ISNU-L-ISN-50 (DQ066167), isolate ISNU-L-ISNU-51 (DQ066183), isolate ISNU-cluster-52 (DQ066136), isolate IS-18-24-clu513 (DQ065897), ISAC-1 (AY674272), *I. ricinus* ferritin (AF068224), *I. scapularis* ferritin (AY277906), *I. ricinus* metalloprotease (MP) (AJ269650), *I. scapularis* MP (AY264367), *I. pacificus* MP (AY674268), *I. ricinus* ribosomal protein S18 (RP S18) (AY342352), *I. scapularis* RP S18 (CA763772), *I. ricinus* calreticulin (AY395272), *I. scapularis* calreticulin (AY395273), *I. pacificus* calreticulin (AY395269), *I. ricinus* actin (AJ889837) and *I. scapularis* actin (AF426178).

Construction of mammalian expression vectors encoding IRACs

IRAC I and IRAC II cDNAs obtained by the RT–PCR approach described above were used as templates to amplify IRAC I and II ORFs. Amplifications of IRAC I were performed with the sense primer 5′-CCGGATCCATGAAGACTGCGCTGAC-3′ containing a 5′ *Bam*HI site and nucleotides 1–17 (nucleotides 1–3 encode the initiation methionine) and either the reverse primer 5′-CCCTCGAGTCATGGGATGGCCTCAGGTT-3′ corresponding to the ORF 3′ end (nucleotides 535–555), the stop codon, and a *Xho*I site or the reverse primer 5′-CCCTCGAGTGGGATGGCCTCAGGTTGAG-3′ corresponding to the 3′ end of the ORF (nucleotides 533–552) and a *Xho*I site. Similarly, amplifications of IRAC II were performed with the sense primer 5′-CCGGATCCATGAGGACTGCGCTGAC-3′ containing a 5′ *Bam*HI site and nucleotides 1–17 and either the reverse primer 5′-GGCTCGAGTCAGGCGATGGCTCTAA-3′ corresponding to the ORF 3′ end (nucleotides 520–537), the stop codon, and a *Xho*I site, or the reverse primer 5′-GGCTCGAGGGCGATGGCTCTAAGTT-3′ corresponding to the 3′ end of the ORF (nucleotides 517–534) and a *Xho*I site. The four PCR products were subcloned into pGEM-T easy (Promega), resulting in pGEM-T-IRAC (I or II) with or without stop codon. Inserts were controlled by sequencing. Fragments encoding IRAC I or II were excised from pGEM-T-IRAC (I or II) by *Bam*HI/*Xho*I digestion and ligated into pcDNA4/TO His/Myc vector (Invitrogen) digested by the same endonucleases, resulting in pcDNA4/TO-IRAC I or II His-tagged and pcDNA4/TO-IRAC I or II wild type.

Expression of IRACs in HeLa cells

HeLa cells (ATCC, CCL-2) were grown in RPMI 1640 medium (Gibco, Merelbeke, Belgium) supplemented with 2% (v/v) penicillin-streptomycin (Gibco) and 10% (v/v) heat-inactivated fetal calf serum (Biowhittaker, East Rutherford, NJ). Subconfluent HeLa cells were transfected using the Effectene reagent (Qiagen, Venlo, The Netherlands). The following vectors were transfected: pcDNA4/TO-IRAC I or II His-tagged, pcDNA4/TO-IRAC I or II wild type and the pcDNA4/TO empty vector (negative control). Seventeen hours after transfection, cell supernatant was replaced with serum-free culture medium. After an additional incubation period of 24 h, the culture medium was collected, clarified by centrifugation at 200 ***g*** for 10 min and concentrated 20-fold using Centricon Plus-20 (10 kDa cut-off) (Millipore Corp., Billerica, MA). Concentrated supernatants were stored at –80 °C until use.

Antibodies

Mouse antisera, hereafter called anti-IRAC I and anti-IRAC II were raised against IRAC I and IRAC II His-tagged molecules expressed in HeLa cells as described above. In addition, two mouse mAb specific of IRAC I (mAb I1a) or IRAC II (mAb I2a) were also produced. mAb I1a and mAb I2a are both IgG1. mAb 198 (Serotec, Oxford, UK) raised against rabbit CD11b is an IgG1 and was used as isotypic control for mAb I1a and mAb I2a. Rabbit sera raised against human C3 (anti-C3) (Serotec) and factor B (fB) (anti-fB) (DakoCytomation, Glostrup, Denmark) were also used in this study.

Anticomplement assays

The ability of IRAC I and IRAC II to inhibit the alternative pathway of the complement system was tested using the AH50 assay as described elsewhere (Coligan *et al.*, 1992). This assay relies on the lysis of unsensitized rabbit erythrocytes (E_{rab}) by human serum in a buffer containing the calcium chelator EGTA, thus preventing the activation of the calcium-dependent classical pathway. Briefly, 50 µl of E_{rab} (2×10^8 cells per ml in ice-cold gelatin/veronal-buffered saline with $MgCl_2$ and EGTA (GVB/Mg EGTA)) were added on ice to 12.5 µl of human serum and increasing amounts of the concentrated cell supernatant to be tested. The final volume of each sample was then brought to 150 µl with GVB/Mg EGTA buffer. For background and total lysis samples, 100 µl of GVB/Mg EGTA buffer or 100 µl of water were added to the 50 µl of E_{rab}, respectively. After an incubation period of 60 min at 37 °C, 1.2 ml of ice cold 0.15 M NaCl was added to each sample to stop haemolysis. After centrifugation at 1250 ***g*** for 10 min at 4 °C, the supernatant was collected and its optical density measured at 412 nm (OD412). Relative haemolysis of each sample was calculated as follow: (test sample OD412 – background sample OD412)/(total lysis sample OD412 – background sample OD412). The ability of IRAC I and IRAC II to inhibit the classical pathway of the complement system was tested using the CH50 assay as described in detail elsewhere (Coligan *et al.*, 1992).

Immunoblotting

Concentrated cell supernatants were analysed by immunoblotting as described previously (Gillet *et al.*, 2005). Anti-IRAC I (diluted 1 : 1000) and anti-IRAC II (diluted 1 : 5000) were used as primary antibodies. Peroxidase-conjugated rabbit immunoglobulin anti-mouse immunoglobulins (diluted 1 : 10 000, Dako) were used as secondary antibody.

Enzyme-linked immunosorbent assays for measuring C3 and fB deposition

The ability of the IRAC I and II molecules to act on C3 and fB during alternative pathway activation was tested using an ELISA assay as described elsewhere with minor modifications (Valenzuela *et al.*, 2000). Briefly, 96 well plates were coated with 50 µl of 0.1% (w/v) agarose in water. To test the ability of IRACs to prevent C3 or fB binding to the plate, 0.5 µl of concentrated cell supernatant containing IRACs was incubated in a 50 µl final volume of 20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EGTA, 2 mM $MgCl_2$ (HEM)

containing 10% human serum for 30 min at 37 °C. For blank control, $MgCl_2$ and EGTA were substituted by 10 mM EDTA, thus inhibiting complement activation. After five washes with 200 μl of HEM supplemented with 10 mg/ml of bovine serum albumin (HEMB), C3b and fB bound plates were revealed by ELISA. Anti-C3b (diluted 1/2500) and anti-fB sera (diluted 1/250) used as primary antibodies were incubated for 1 h at 37 °C in HEMB. After extensive washing with HEMB, samples were incubated for 1 h at 37 °C with peroxidase-conjugated IgG goat antirabbit immunoglobulins (diluted 1 : 200) (Dako Cytomation), as secondary antibody. Quantification of peroxidase activity was then performed as described elsewhere (Valenzuela *et al.*, 2000). To test the ability of IRACs to uncouple previously bound C3 and fB, 50 μl of HEM containing 10% human serum was incubated for 75 min at 37 °C to allow C3b and fB binding to plate. After three washes with HEM, increasing volumes of concentrated supernatant containing IRACs were incubated in a 50 μl final volume of HEM containing 10% heat inactivated human serum for 30 min at 37 °C. After five washes with 200 μl of HEMB, samples were treated as described above for relative quantification of C3b and fB by ELISA.

Indirect immunofluorescent staining

Indirect immunofluorescent stainings were performed on three types of samples: cryosections of ticks, microdissected tick salivary glands and cell monolayers grown on glass coverslips. Unfed or 4-day-fed ticks were embedded in tissue-tek compound (Sakura, Zouterwoude, The Netherlands) and frozen in liquid nitrogen. Twenty micron thick parafrontal sections were then performed through the entire tick and bound to poly L-lysin-coated slides. All types of samples were fixed in phosphate-buffered saline (PBS) (PBS: 3 mM KCl, 1.5 mM KH_2PO_4, 0.14 M NaCl, 6.5 mM Na_2HPO_4, pH 7.2) containing 4% (w/v) paraformaldehyde (Merck, Whitehouse Station, NJ) for 15 min on ice and then 30 min at room temperature. After washing with PBS, the samples were then permeabilized in PBS containing 0.1% (w/v) NP40 (Fluka, St. Louis, MO) for 20 min at 37 °C. Immunofluorescent stainings (incubation and washes) were performed in PBS containing 10% (v/v) fetal calf serum (PBSF). The samples were incubated at 37 °C for 45 min with anti-IRAC I or II sera (diluted 1/50), preimmune mouse sera (diluted 1/50, negative control), mAb I1a (diluted 1/100), mAb I2a (diluted 1/500) and mAb 198 (diluted 1/100), as primary antibodies. After three washes, the samples were incubated at 37 °C for 30 min with Alexa488-conjugated $F(ab')_2$ goat antimouse IgG (Alexa-GAM) 10 μg/ml (Molecular Probes, Merelbeke, Belgium) as secondary conjugate. After three washes with PBSF and a final wash with PBS, samples were mounted as described elsewhere (Vanderplasschen *et al.*, 2000). For tick cryosections, cell nuclei were counter-stained just before mounting by incubation in PBS containing 0.1% (w/v) TO-PRO-3 iodide (Molecular Probes) for 10 min.

Confocal microscopy analysis

Confocal microscopy analyses were performed with a TCS SP confocal microscope (Leica) as described previously (Vanderplasschen *et al.*, 2000).

Statistical analysis

Statistical comparisons were assessed by a two-way analysis of variance (ANOVA II). When F ratios were significant ($P < 0.05$), the method of Scheffe's post hoc tests was used.

Sequence analysis

Sequences were aligned using CLUSTALW (Thompson *et al.*, 1994), with subsequent minor manual adjustment; sites with a gap in any sequence were then excluded. Phylogenetic trees were inferred by the Bayesian method (Huelsenbeck & Ronquist, 2001) implemented in the parallel version of MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003; Altekar *et al.*, 2004), using the Jones, Taylor and Thornton (Jones *et al.*, 1992) model of amino acid replacements with a gamma distribution of rates among sites (Yang, 1993). The analysis was run for 1 million generations, with a burn-in of 25%; estimated sample sizes from the Tracer program (http://evolve.zoo.ox.ac.uk/software.html/id=tracer) were greater than 2785.

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