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Identification of a functional Antigen5-related allergen in the saliva of a blood feeding insect, the tsetse fly

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ABSTRACT

Our previous screening of a *Glossina morsitans morsitans* λgt11 salivary gland expression library with serum of a tsetse fly exposed rabbit identified a cDNA encoding Tsetse Antigen5 (TAg5, 28.9 kDa), a homologue of Antigen5 sting venom allergens. Recombinant TAg5 was produced in *Sf*9 cells in order to assess its immunogenic properties in humans. Plasma from a patient that previously exhibited anaphylactic reactions against tsetse fly bites contained circulating anti-TAg5 and anti-saliva IgEs. In a significant proportion of plasma samples of African individuals, TAg5 and saliva binding IgEs (respectively 56 and 65%) can be detected. Saliva, harvested from flies that were subjected to TAg5-specific RNA interference (RNAi), displayed significantly reduced IgE binding potential. Allergenic properties of TAg5 and tsetse fly saliva were further illustrated in immunized mice, using an immediate cutaneous hypersensitivity and passive cutaneous anaphylaxis assay. Collectively, TAg5 was illustrated to be a tsetse fly salivary allergen, demonstrating that Antigen5-related proteins are represented as functional allergens not only in stinging but also in blood feeding insects.

1. Introduction

Tsetse flies (*Glossina* sp.) are obligate blood feeding Diptera and the main vectors for human and veterinary parasitic diseases known as African trypanosomiasis. During each blood feeding event, they deposit a complex salivary mixture at the bite site that modulates the host immune system (Caljon et al., 2006a) and supports efficient parasite transmission (Caljon et al., 2006b). Repetitive exposure of hosts to stinging as well as biting insects might result in hypersensitivity reactions as a consequence of specific humoral and cellular immune responses.

Immediate type (type I) hypersensitivity reactions, inflicted by bites of different arthropod species mostly remain topical, although hypersensitivity reactions with systemic manifestations such as atopy and anaphylaxis occasionally occur (Peng et al., 2004). In the case of exposure to *Glossina* bites, immediate hypersensitivity reactions have been described in exposed rabbits (Ellis et al., 1986) while anaphylactic complications were reported in a laboratory tsetse fly worker (Stevens et al., 1996). The IgE inducing potential has been described for the saliva of several blood sucking arthropods including tsetse flies (Ellis et al., 1986; Caljon et al., 2006a), mosquitoes (*Aedes*, *Anopheles*, *Culex*) (Chen et al., 1998; Peng et al., 2004), sand flies (*Lutzomyia*) (Gomes et al., 2002) and ticks (*Ixodes*, *Boophilus*) (Ogden et al., 2002; Kashino et al., 2005). Beside the involvement of saliva-specific IgEs, several downstream modulators of the hypersensitivity reaction have been described. Mast cell degranulation can be potentiated by adenosine (Bochenek et al., 2008) that might be selectively converted by adenosine deaminases, demonstrated to be present in the saliva of tsetse flies (Li and

Abbreviations: TAg5, Tsetse Antigen5; HAT, human African trypanosomiasis; *Sf*9 cells, *Spodoptera frugiperda* 9 cells.

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Aksoy, 2000), sand flies (Kato et al., 2007), and mosquitoes (Ribeiro et al., 2001). Sequestration of the vaso-active histamine by nitrosyl-heme proteins (nitrophorins) or proteins with a lipocalin fold has been documented for respectively *Rhodnius* (Ribeiro and Walker, 1994) and *Rhipicephalus* (Paesen et al., 1999). In contrast, a functional histamine release factor has been found in saliva of dog ticks (*Dermacentor variabilis*) (Mulenga et al., 2003). The salivary presence of direct mast cell activators was also suggested by the observation that *Anopheles* bites activate mast cells in previously unexposed animals (Demeure et al., 2005). Collectively, different blood feeding arthropod species apparently modulate the onset of hypersensitivity reactions in naive and sensitized hosts.

In the case of *Glossina morsitans morsitans*, salivary transcriptome analysis revealed the presence of an Antigen5 (Ag5)-related cDNA (Li et al., 2001). The encoded protein, Tsetse Antigen5 (TAg5, 28 kDa), was illustrated to be present as secreted protein in tsetse saliva by using mass spectrometry analyses (Haddow et al., 2002). TAg5 belongs to the CAP protein family that includes cysteine-rich secretory proteins, Ag5 proteins of insects and pathogenesis-related protein 1 of plants (Schreiber et al., 1997). Antigen5 proteins are reported to be allergens of the sting venom of hornets, wasps and fire ants and are thought to be involved in triggering acute hypersensitivity responses in mammals (King and Spangfort, 2000; Henriksen et al., 2001). Proteins related to these Ag5 proteins are also expressed in the salivary glands of several blood sucking insects including tsetse flies, sand flies, stable flies and mosquitoes (Li et al., 2001; Valenzuela et al., 2003; Ribeiro et al., 2004; Ameri et al., 2008), but their relation to allergic reactions has not yet been documented. Here, we identify TAg5 as a functional tsetse fly salivary allergen that has predicted structural similarities with the *Vespula vulgaris* venom allergen v 5 and demonstrate its potential to sensitize mice and humans and to induce hypersensitivity reactions in mice.

2. Material and methods

2.1. Animals

6–8 Weeks old in-house bred female Toll-like receptor-2/4 (TLR-2/4) deficient mice were used for the allergenicity experiments.

Tsetse flies (*G. m. morsitans*) were maintained at the Institute of Tropical Medicine Antwerp as described elsewhere (Van Den Abbeele et al., 2007). Experiments were approved by VUB and ITM animal ethics committees.

2.2. Tsetse fly saliva isolates

Saliva was harvested as described earlier (Caljon et al., 2006a,b) and protein concentrations were determined with the BCA kit (Pierce Biotechnology).

2.3. cDNA sequence analysis and structure prediction

Full-length cDNA sequences encoding TAg5, previously described by Li et al. (2001), were identified from a λgt11 salivary gland expression library (Van Den Abbeele et al., 2007). Signal peptide cleavage site prediction was performed using the SignalP 3.0 server. Theoretical molecular weights were determined using the ProtParam tool on the ExPASy server (www.expasy.org) from the Swiss Institute of Bioinformatics. Amino acid sequences were subsequently submitted to the SDAP (Structural Database of Allergenic Proteins, Ivanciuc et al., 2002) and structure prediction was based on comparison of Hidden Markov Models (http://toolkit.tuebingen.mpg.de/hhpred). PDB-files were generated using a program for comparative protein structure modelling (http://salilab.org/modeller/). 3D models were rendered using the DeepView/Swiss-PdbViewer (http://www.expasy.org/spdbv/).

2.4. In vivo RNA interference (RNAi)

The RNA interference (RNAi) method was adopted for the selective silencing of TAg5 expression and was based on the injection of a TAg5-specific double stranded RNA (dsRNA) molecule. As a negative control, dsRNA was generated from the pBluescriptSK(+) plasmid. Templates for dsRNA production by *in vitro* transcription (IT) were generated by PCR, incorporating opposing T7 promotors at both ends of the amplicon. This allowed an *in vitro* transcription (IT) reaction from 1 template, yielding both a sense and an antisense gene-specific RNA molecule. To generate the IT templates for the production of the mock dsRNA (pBluescript dsRNA: 451 bp) and TAg5-specific dsRNA (TAg5 dsRNA: 485 bp), specific primer pairs were constructed with Clone Manager Professional Suite 6.00 or the web-based E-RNAi program (http://www.dkfz.de/signaling2/e-rnai/): *TAg5(IT) sense* (5′-TAATACGACTCACTATAGGGAGTGGGTGGAGATTATTGCG-3′), *TAg5(IT) antisense* (5′-TAATACGACTCACTATAGGGCGACCATGACTGTGAAATGG-3′), *PBlue(IT) sense* (5′-GCTAATACGACTCACTATAGGGAGAGGCACCTATCTCAGCGATCT-3′) and *PBlue(IT) antisense* (5′-GCTAATACGACTCACTATAGGGAGACACTGCGGCCAACTTACTTC-3′). IT templates were amplified from respective pBluescriptSK(+) and a *tag5* containing plasmid. The PCR amplicons were column purified (Qiagen) and eluted in nuclease-free water. The *in vitro* transcription was performed using the Megascript RNAi kit (Ambion), following the manufacturer's instructions. Briefly, the T7 RNA polymerase reaction was performed from 2 μg template for 5 h at 37 °C. Duplex yield was maximized by denaturing IT reaction products at 75 °C for 5 min, followed by a gradual cool-down to room temperature. The dsRNA was further enzymatically purified by digestion of single stranded RNA (ssRNA) and template DNA for 1 h at 37 °C using the RNAse and DNAse I provided in the kit. This was followed by column purification and elution of the dsRNA in 2× 100 μl prewarmed (95 °C) nuclease-free water. dsRNA samples were further concentrated in a Speedvac (DNA Speedvac, Savant), at maximum centrifugation speed and drying rate. Samples were brought to an equal concentration of 4–6 μg/μl as determined spectrophotometrically, followed by analysis on a 1% agarose gel. dsRNA samples were stored at −20 °C until just prior to injection.

Tsetse flies, 48 h after the last blood meal, were briefly anaesthetized by cold shock and injected with 15 μg dsRNA. Intrathoracic injections were performed under a binocular microscope, using a 5 μl Hamilton 75RN microsyringe with gauge 30 removable electrotapered needles.

2.5. Evaluation of transcriptional and translational RNAi silencing efficiencies

In order to evaluate the RNAi silencing efficiency, 10 pairs of tsetse fly salivary glands were isolated at eight days after the intrathoracic injection of the dsRNA. Saliva was harvested by centrifugation and used for evaluating the silencing efficiency at the protein level. Salivary glands in the pellet were disrupted using Teflon homogenizers and total RNA was extracted using Trizol reagent (Gibco–Invitrogen). 1 μg of total RNA was reverse-transcribed using oligo(dT) and Superscript II reverse transcriptase (Gibco–Invitrogen) following the manufacturer's recommendations. Quantitative real-time PCR was performed in a Bio-Rad (Hercules, CA) iCycler, with Bio-Rad iQ SYBR Green Supermix. Used primers were: *Tsetse Antigen5 (TAg5) sense* (5′-GATTATTGCGGCTTGTGCGA-3′), *TAg5 antisense* (5′-ATTGGTGGTGGCGTAATTGC-3′), tsetse salivary gland protein 2 (Tsal2) sense (5′-CCAAGAACTGGCTGACCAA-3′), *Tsal2 antisense* (5′-CTGCCAGCAGATTGTGTAAC-3′),

tsetse salivary gland growth factor 1 (TSGF-1) sense (5′-CGGTTG TAAATCCGAATCTGT-3′), *TSGF-1 antisense* (5′-TTTGCTCGTGT CGTCGTATGG-3′) *tubulin sense* (5′-GATGGTCAAGTGCGATCCT-3′), *tubulin antisense* (5′-TGAGAACTCGCCTTCTTCC-3′), *actin sense* (5′-CGCTTCTGGTCGTACTACT-3′) and *actin antisense* (5′-CCGGA CATCACAATGTTGG-3′). The amplicon sizes were respectively 573, 309, 274, 355 and 474 bp. For all primers sets, each PCR cycle consisted of 1 min denaturation at 94 °C, 45 s annealing at 54 °C and 1 min extension at 72 °C. Gene expression was normalized using the Genenorm program.

To evaluate the effect of RNAi on TAg5 translation, harvested salivary proteins were run under reducing and denaturing conditions on a 10% SDS-polyacrylamide gel. Proteins in the gels were stained with Coomassie Brilliant blue. In order to analyze band intensities, dried gels were scanned at 300 dpi in greyscale and saved as TIFF-files. Band intensities were quantified as integrated peak OD measurements using the Imagemaster 1D Elite v3.01 software package (Amersham Pharmacia Biotech). Band intensities were normalized using the TSGF-1 band intensity measurements, followed by the calculation of relative silencing efficiencies as compared to H_2O and control dsRNA treated samples.

2.6. Recombinant TAg5 expression in Spodoptera frugiperda cells

The *tag5* gene including the secretion signal sequence was amplified from a salivary gland cDNA library (Van Den Abbeele et al., 2007) by PCR (Mastercycler personal, Eppendorf) using the *TAg5Forward* (5′-CGCGGATCCACCATGAATTTCGTGCTTGCGACCTTAA GC-3′) and *TAg5Reverse* (5′-TTAATGATATTCGACACCATTTTTGATCC ATTTATTGACC-3′) primers. Following TA-cloning in a pGEM-T vector (Promega), the *tag5* gene was integrated in a pVL1393 vector for the expression in *S. frugiperda* (*Sf9*) insect cells (Invitrogen) using the NcoI and BamHI restriction enzymes (NEB).

The *Sf9* insect cells were grown at 27 °C in SF-900 II serum-free medium (Invitrogen) until monolayered cultures reached a cell density of 2×10^6 cells in 25 cm^2 culture flasks. The pVL1393-*tag5* construct was cotransfected with the BD Baculo-Gold Baculovirus DNA using the BD BaculoGold Transfection Kit (BD Biosciences Pharmingen). The supernatant containing recombinant Baculovirus particles was harvested 72 h post-transfection and further amplified in *Sf9* shaking cultures. After four to seven amplification cycles of four days, the culture supernatant was harvested and proteins precipitated using 60% ammonium sulphate. Pellets were reconstituted in 50 mM phosphate buffer, 50 mM NaCl pH 7.7 before loading onto a DEAE-Sephacel column (5 mL, GE Healthcare) for a negative selection for TAg5. The collected flow-through was dialysed against 50 mM phosphate buffer, 50 mM NaCl pH 6 and applied onto a HiTrap SF FF column (1 mL, GE Healthcare) connected to an Äkta explorer chromatography system P-960. The retained proteins were eluted in LPS-free conditions in a linear gradient to 1 M NaCl in 50 mM phosphate buffer pH 6.0. The TAg5 protein eluted in a single peak at 350 mM NaCl. Purified TAg5 was analysed on silver stained (SilverQuest™ Silver Stain Kit, Invitrogen) 12% SDS-polyacrylamide gels. TAg5 identity and purity were analysed by means of western blot with an anti-TAg5 polyclonal rabbit serum and by MALDI-TOF mass spectrometry.

2.7. TAg5 and saliva immunizations

Twelve TLR-2/4$^{-/-}$ mice per group were sensitized either by subcutaneous inoculation of recombinant TAg5 or saliva extracts (without adjuvant) or by exposure to tsetse fly bites. Subcutaneous immunization was performed weekly with respectively 5 μg, 2 μg and four times 1 μg TAg5 or saliva in PBS. Natural saliva immunizations were performed by allowing 5 flies per mouse to feed weekly over a period of 6 weeks. One week after the last boost, sera were prepared from tail vein blood samples.

2.8. TAg5 and saliva-specific IgE antibody titers

Mouse anti-TAg5 and anti-saliva IgE antibody responses were assessed in solid phase ELISA. For this purpose, Immunosorb plates (Nunc) were coated with 200 ng antigen (recombinant TAg5 or saliva collected from tsetse flies) per well in 0.1 M $NaHCO_3$ (pH 8.3) and blocked with 10% FBS in PBS. Serial ½ serum dilutions were applied to both antigen and FBS coated wells followed by specific IgE detection, using a peroxidase-conjugated rat anti-mouse IgE antibody (SouthernBiotech).

2.9. Assessment of immediate cutaneous hypersensitivity to TAg5 and tsetse saliva

Hypersensitivity to TAg5 and saliva exposure was assessed using Evans blue extravasation as a read-out for mast cell degranulation, according to the procedure of Seitzer et al. (2003). Intradermal challenge was performed with 50 and 100 ng of respectively saliva and TAg5 in 25 μl 0.9% NaCl. Histamine releasing factor compound 48/80 (1000 and 500 ng, Sigma) and 0.9% NaCl served as respective positive and negative controls. Evans blue extravasation was monitored within 15 min after allergen challenge and illustrated using digital photography.

2.10. Passive cutaneous anaphylaxis

Naive serum and immune anti-TAg5 or anti-saliva sera from the best responder mice were pooled and ½ diluted in PBS. 25 μl of the diluted serum was injected intradermally in the ear pinna (immune serum in the left ear, naive serum in the right ear of the same mouse) for local sensitization. 24 h later, mice were injected intravenously with Evans blue, anesthetized and subcutaneously challenged in both ears with 200 ng TAg5 or saliva in 25 μl 0.9% NaCl. Dye extravasation was monitored within 1 h after challenge, followed by euthanasia and mincing of the dissected ears in 300 μl formamide. After overnight incubation at −80 °C, formamide extracts were filtered and optical densities measured at 630 nm.

2.11. Anti-TAg5 and anti-saliva IgE immune screening of human plasma samples

In order to determine human IgE responses to tsetse fly saliva and TAg5, we examined a panel of 177 plasma samples from individuals recruited in tsetse fly endemic regions of Tororo and Soroti (Uganda) as part of a study described elsewhere (Maclean et al., 2005). In addition, we used a panel of 26 plasma samples that were collected as a continuation of a previous study in Ndombo (North Senegal) that is not a tsetse fly endemic area (Polman et al., 2001). 17 European plasma samples were incorporated as negative controls. An additional plasma sample was obtained from a laboratory worker that previously developed an anaphylactic reaction against tsetse fly bites (Stevens et al., 1996). The use and collection of the Ugandan plasma samples followed protocols approved by Ethical Committees in Uganda (Ministry of Health) and the UK (Grampian Research Ethics Committee). For the Senegalese samples, ethical clearance was obtained from the Ethical Commission of the European Special Program for Operational and Integrated Research (ESPOIR) within the Senegalese Ministry of Health and by the Ethical Commission of the Leiden University Medical Centre. All plasma samples were tested in a TAg5 and total saliva-specific human IgE detection ELISA.

For the ELISA analysis, antigen coating conditions were the same as described above. Human plasma samples were 1/200 diluted in PBS with 10% FBS. Plasma samples were incubated for 1 h in the ELISA plate prior to detection of TAg5 and saliva-specific IgEs using a peroxidase-conjugated rat anti-human IgE (ε-chain specific, Sigma) and 3,3′,5,5′-tetramethylbenzidine substrate. ELISA results were expressed as the differences between the measured O.D.s (λ: 450 nm) and the average O.D. of the European negative control samples. Comparison of the responses to the antigen (either TAg5 or saliva) coated wells and the FBS block coated wells allowed subdivision into 2 responder types: (i) an antigen-specific response ($\Delta O.D. > 0.05$) more than double of the ΔO.D. on the overcoat (specific) and (ii) no significant binding to the antigen as compared to the FBS coat (negative).

For western blot analysis, 75 μg saliva was separated on pre-casted 10% SDS-polyacrylamide gels with a single big slot (Invitrogen), followed by electrotransfer to a nitrocellulose membrane (HyBond C, GE Healthcare). Antigen detection was performed with 1/200 diluted human plasmas in PBS with 10% FBS, followed by a detection using peroxidase-conjugated rat anti-human IgE (Sigma) and chromogenic substrate.

2.12. Graphs and statistical analysis

All graphs were prepared by the use of the GraphPad Prism 4.0 software (GraphPad Software). The same software was used for statistical analysis (two-tailed unpaired *t*-tests, Kruskal–Wallis one-way ANOVA) of the data. Data are represented as mean ± SEM.

3. Results

3.1. Tsetse fly specific IgE responses in plasma from an anaphylaxis patient

In 1996, a laboratory worker was reported to have displayed anaphylactic reactions upon tsetse fly bites (Stevens et al., 1996). We obtained plasma samples taken in 2005 and 2008, and analysed these by ELISA for anti-saliva IgE titers. Relatively higher anti-saliva IgE antibodies were detected in the 2005 sample (Fig. 1A). The subject has declared to be unexposed to tsetse fly bites for at least one year before the last sample was taken, suggesting that seroconversion is responsible for the currently undetectable anti-saliva IgE levels.

Qualitative assessment of the anti-saliva IgE response by western blot illustrated reactivity against the 43–45 and 28 kDa protein bands in total tsetse fly saliva (Fig. 1B, lane 2). Nearly undetectable IgE binding to the 64–66 and 57–58 kDa protein bands was observed (Fig. 1B, arrows lane 2), while other salivary constituents (Fig. 1B, lane 1) remained unrecognized by IgEs.

3.2. cDNA analysis of the putative 28 kDa allergen

A cDNA, Tsetse Antigen5 (*tag5*), that corresponds to the 28 kDa IgE-binding protein, was picked up during our immune screening of a *G. m. morsitans* λgt11 salivary gland expression library (Van Den Abbeele et al., 2007). The full-length *tag5* cDNA encodes a protein of 259 amino acids with 13 cysteine residues and a predicted molecular weight of 28,925 Da. Signal peptide prediction suggests most likely cleavage between amino acids 19 and 20. As such, the secreted protein has a probable molecular weight of 27,052 Da.

The TAg5 amino acid sequence displays a significant degree of similarity with the CAP family (Li et al., 2001), including the cysteine-rich secretory proteins, Antigen5 allergens of insects and pathogenesis-related-1 proteins of plants. Comparison of the TAg5 sequence with sequences available on the Structural Database of Allergenic Proteins (SDAP), suggested clustering with allergens of the Pfam A PF00188 family that exclusively includes insect allergens such as the Ves v 5, a venom allergen from *V. vulgaris* [PDB: 1qnx, GenBank accession no. M98858 (Henriksen et al., 2001)]. Structure prediction based on comparison of Hidden Markov Models, proposes a very similar structure for TAg5 as Ves v 5 with a characteristic α–β–α sandwich fold, consisting of a three-stranded anti-parallel β-sheet sandwiched between an α-helix and a layer of another 2 α-helices (Fig. 2).

3.3. Production and purification of TAg5 from Sf9 cells

Recombinant TAg5 was produced in an *Sf9* insect cell expression system, yielding nearly homogenous preparations. Purity was assessed by SDS-PAGE followed by protein detection with silver salts (Fig. 3A). The TAg5 identity was confirmed by western blot and by MALDI-TOF mass spectrometry. The presence of a low molecular

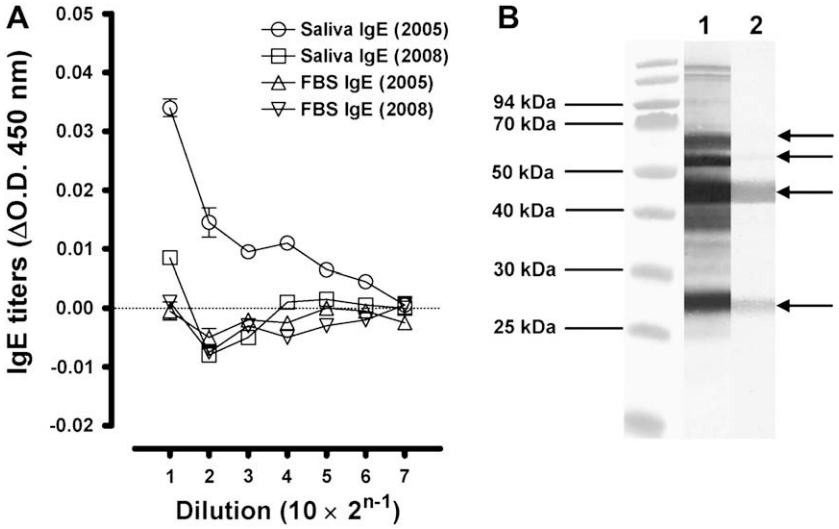

Fig. 1. (A) ELISA-based detection of anti-FBS and anti-saliva IgE responses in plasmas taken in 2005 and 2008 from a patient that exhibited anaphylactic reactions to tsetse fly bites. (B) western blot analysis to assess the specificity of the plasma IgEs in the same anaphylactic patient (strip 2, positive bands are indicated with arrows). Strip 1 shows the saliva protein profile revealed by rabbit anti-saliva IgG detection.

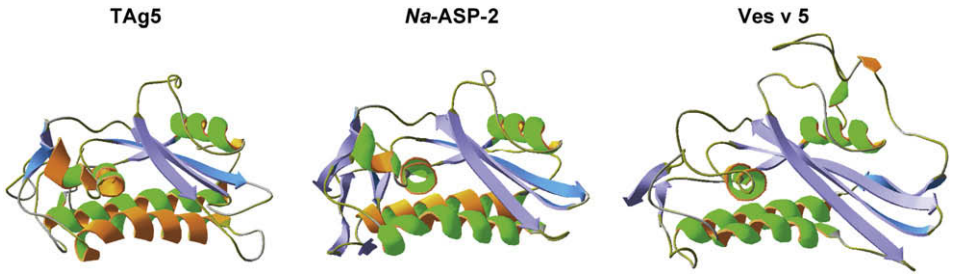

Fig. 2. 3D model of the predicted TAg5 structure as an α–β–α sandwich protein. Structure prediction was based on the resolved structures of Na-ASP-2, a pathogenesis-related protein 1 of the nematode *Necator americanus* [PDB: 1U53, GenBank accession no. AY288089 (Asojo et al., 2005)] and the Ves v 5, a venom allergen from *Vespula vulgaris* [PDB: 1qnx, GenBank accession no. M98858 (Henriksen et al., 2001)].

weight TAg5 degradation product and a minor contamination with the high molecular weight *Bombyx mori* (silk moth) translation elongation factor 2 (EF2) resulting from the Baculovirus expression system, was detected by mass spectrometry and was visible on the silver stained SDS-polyacrylamide gel (Fig. 3A).

The presence of TAg5 as soluble protein in saliva was confirmed by western blot, using rabbit IgGs that were raised against recombinant TAg5 (Fig. 3B). Inversely, rabbit anti-saliva IgG responses were able to bind to recombinant TAg5 in immunoblots (data not shown).

3.4. TAg5 exhibits sensitizing properties in human

Recombinant TAg5 was used as coating antigen in a human IgE ELISA, to analyze the presence of TAg5-specific IgEs in the plasma taken in 2005 from the anaphylaxis patient. TAg5-specific IgEs were apparent in the patient's plasma (Fig. 4A), indicating the sensitizing properties of TAg5 in humans.

We further evaluated the presence of anti-TAg5 IgE responses in 1/200 diluted plasma samples from S.E. Ugandan individuals ($n = 177$), inhabiting the eastern part of the African tsetse belt. 56 and 65% of these plasma samples scored positive ($P < 0.001$) for the presence of TAg5 and saliva binding IgEs (Fig. 4B), with a linear correlation between the recorded IgE titers ($r^2 = 0.62$, Fig. 4C). We next evaluated the responses in plasma samples ($n = 26$) collected outside the tsetse endemic area in North Senegal, and also detected increased IgE binding to the antigenic coats (TAg5 and saliva) as compared to the overcoat (FBS) (data not shown). Plasma samples from European individuals ($n = 17$) did not display an overall higher response to the antigenic coats than the blocking antigen ($P > 0.05$). However, our criterion still identified 24 and 12% positive responders for respectively TAg5 and saliva within the tested European plasmas (Fig. 4B).

3.5. In vivo functional analysis of TAg5 by RNA interference (RNAi)

RNAi was applied for *in vivo* functional analysis of TAg5 by intrathoracic injection of 15 μg dsRNA per fly. This injection dose was very well tolerated (>95% tsetse fly survival) and resulted in specific TAg5 silencing. At day 8 after injection, an approximate 93% inhibition at the mRNA level was achieved (Fig. 5A) while translation was less affected with an observed protein reduction of 56% (Fig. 5A and 5B). No significant effects of the TAg5 silencing on feeding times, blood meal weights and survival were recorded (data not shown). Next, TAg5-specific and control RNAi treated saliva samples were used as antigenic coats in an IgE detection ELISA. As a result of TAg5 silencing, significantly reduced IgE binding was observed for both African ($P < 0.0001$) and European plasma samples ($P = 0.002$) (Fig. 5C).

3.6. TAg5 exhibits sensitizing and allergenic properties in mice

To illustrate the correlation between anti-TAg5 IgE responses and TAg5-induced mediator release, four groups of mice ($n = 12$/group) were used for sensitization and allergenicity studies: control (injected with PBS), recombinant TAg5 and saliva immunized as well as tsetse fly exposed mice. ELISA analysis indicates that immunization with 6 decreasing doses of either TAg5 or saliva is sufficient to raise TAg5-specific IgE responses (Fig. 6A). Immunized mice were subsequently subjected to intradermal challenge with 50 and 100 ng of recombinant TAg5 (spots 1 and 2) or tsetse saliva (spots 3 and 4), followed by monitoring the local mast cell/basophil degranulation reactions using Evans blue extravasation as read-out (Fig. 6B). The 48/80 mast cell activating peptide (spots B and C) and 0.9% NaCl (spot A) were used as respective positive and negative controls. Challenge of tsetse fly exposed or saliva-immunized mice with recombinant TAg5 yielded mild hypersensitivity reactions

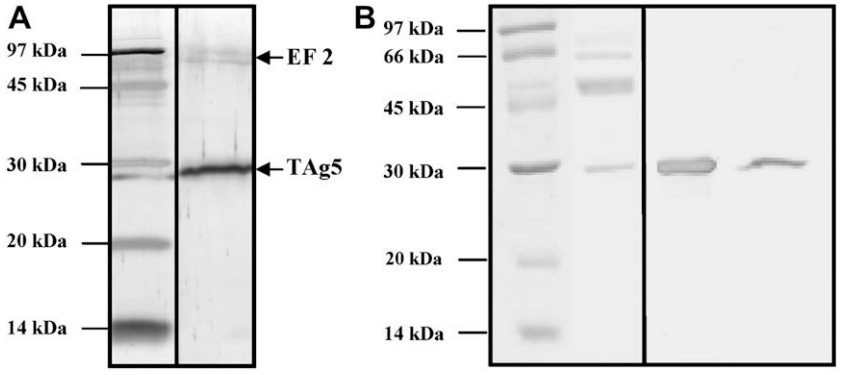

Fig. 3. (A) Silver stained protein profile of purified *Sf*9 insect cell-derived recombinant TAg5 separated on a 12% SDS-polyacrylamide gel [lane 1: LMW protein ladder (Amersham Biosciences), lane 2: purified recombinant TAg5]. The TAg5 and contaminating EF2 protein bands are indicated with arrows. (B) Coomassie-stained 12% SDS-polyacrylamide gel [lanes 1–2: LMW protein ladder (Amersham Biosciences), *G. m. morsitans* saliva extract] and the western blot analysis with mouse anti-TAg5 serum [lanes 3–4: *G. m. morsitans* saliva extract, recombinant TAg5 in the supernatant of transfected *Sf*9 cells after the 6th virus amplification].

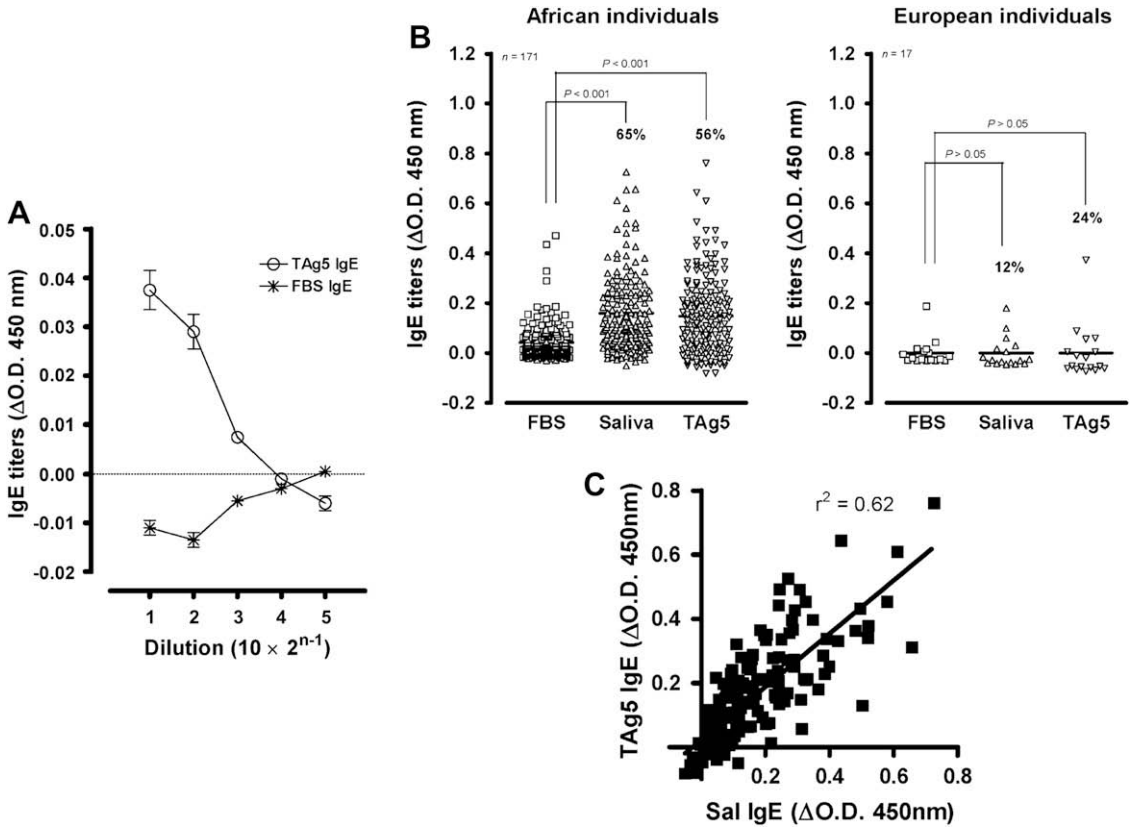

Fig. 4. The sensitizing properties of TAg5 in humans: (A) ELISA-based detection of anti-TAg5 and anti-FBS IgE responses in plasma taken in 2005 from a patient that previously exhibited anaphylactic reactions to tsetse fly bites. (B) Anti-FBS, anti-saliva and anti-TAg5 IgE responses in 177 African individuals and 17 European individuals. Represented data are Δ O.D.s (O.D. – average O.D. from European controls). Values above each data set represent percentages positive responders, using a two times higher response on antigen versus FBS blocking antigen as cut-off. (C) Correlation between detected anti-saliva and anti-TAg5 IgE titers (Δ O.D.s) in the 177 African plasmas.

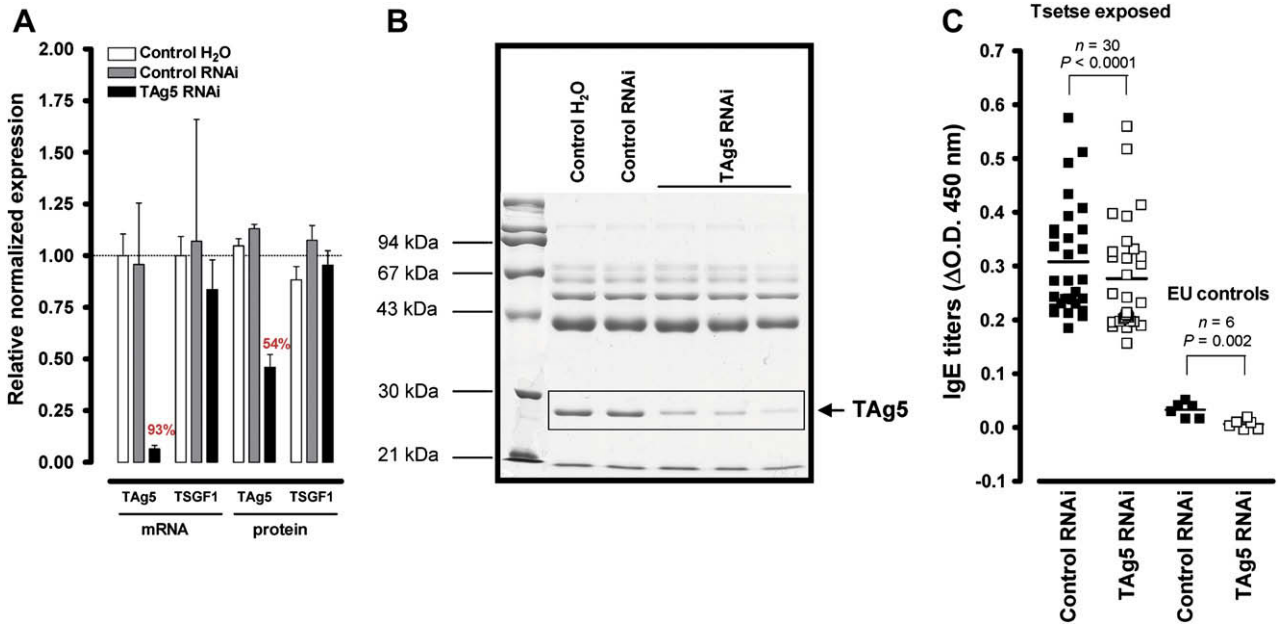

Fig. 5. TAg5-specific RNAi: (A) relative normalized TAg5 and TSGF-1 mRNA and protein levels at day 8 after intrathoracic injection of 15 μg dsRNA (TAg5 RNAi, control RNAi, H_2O control) (B) Coomassie-stained protein profiles of saliva harvested from H_2O control, control RNAi and TAg5 RNAi treated flies. The TAg5 protein band is indicated with an arrow. (C) IgE responses of African (tsetse exposed, $n = 30$) and European individuals ($n = 6$) against total saliva, harvested from control (■) and TAg5 RNAi treated flies (□).

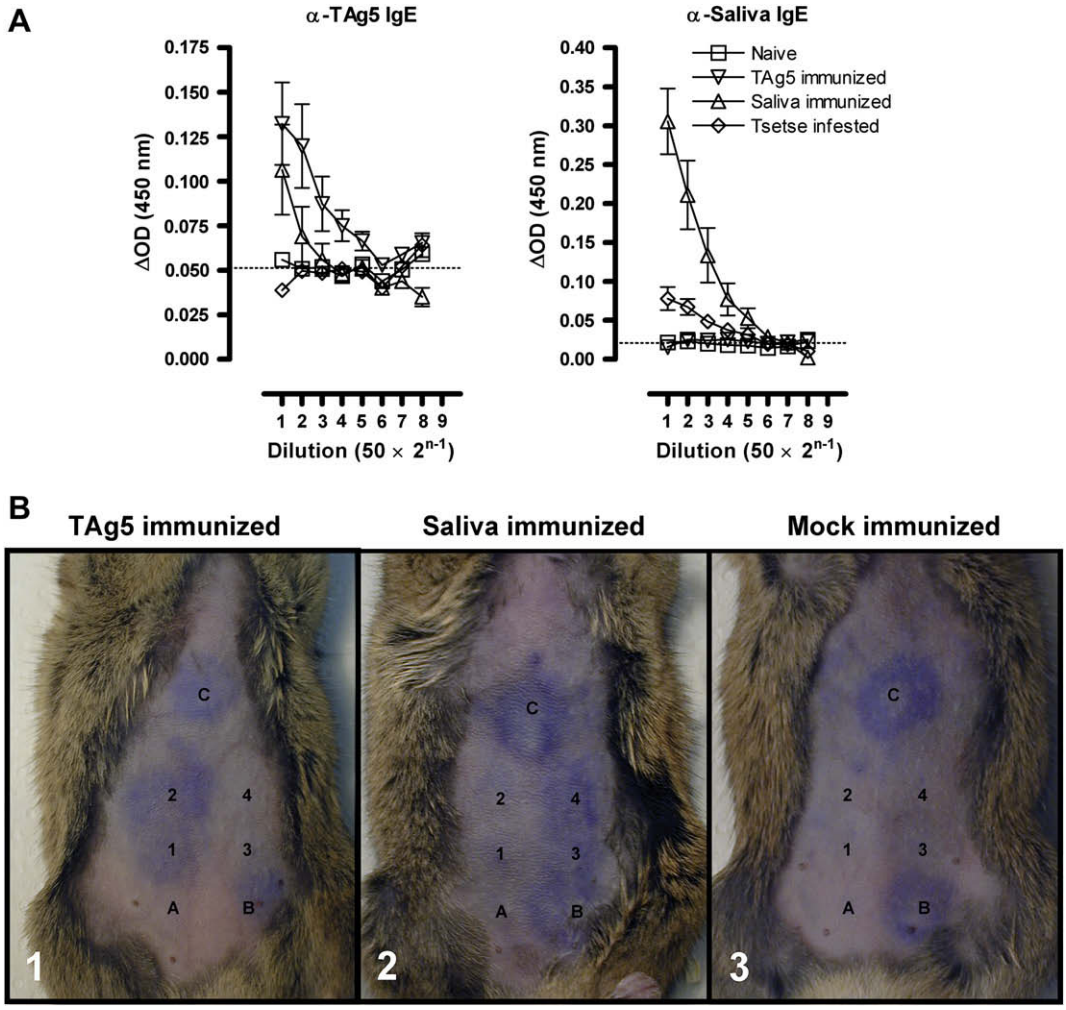

Fig. 6. Sensitizing and allergenic properties of TAg5 in mice ($n = 12$/group): (A) induction of recombinant TAg5 and tsetse saliva-specific IgEs in the different immunization groups [naive (□), TAg5 immunized (▽), saliva immunized (△), exposed to tsetse fly bites (◇)]. (B) Skin allergenicity tests ($n = 6$/group) using Evans blue extravasation as read-out. Pictures are those of representative responder mice that were immunized with recombinant TAg5 (panel 1), saliva (panel 2) or with a placebo (panel 3) and subcutaneously injected with 50 and 100 ng TAg5 (spots 1 & 2), 50 and 100 ng tsetse fly saliva (spots 3 & 4), 0.5 and 1 μg mast cell activating peptide 48/80 (spots B & C) and 0.9% NaCl solution (A).

(Fig. 6B, panel 2), while positive skin tests were strongly apparent upon homologous challenge (Fig. 6B, panels 1 and 2). Saliva (100 and 50 ng) by itself did not induce mast cell degranulation in non-immunized hosts (Fig. 6B, panel 3), suggesting that there are no direct mast cell activators in tsetse fly saliva.

Pooled and diluted naive and immune sera were subsequently used for a passive cutaneous anaphylaxis assay. Anti-TAg5 and anti-saliva immune sera were able to sensitize the ear dermis of non-immune mice to respond to a local challenge with TAg5 and saliva 24 hours later (Fig. 7A and B). Homologous challenge induced the strongest responses as determined by Evans blue quantification in formamide extracts (Fig. 7B).

4. Discussion

Tsetse flies are dipteran insects of a major clinical importance as they are the sole transmitters of human African trypanosomiasis, a parasitic disease better known as sleeping sickness. We have previously shown that immunization against saliva by repeated tsetse fly bites enhances the early onset of a trypanosome infection, suggesting that the parasite benefits from local hypersensitivity reactions for host colonisation (Caljon et al., 2006b). Hypersensitivity reactions induced by tsetse flies or saliva extracts have only been reported in this study, in an experimental rabbit model (Ellis et al., 1986) and in a case study of a laboratory worker that displayed tsetse-induced anaphylactic reactions (Stevens et al., 1996). Plasma taken in 2005 from this patient, who since 1995 still underwent occasional exposure to tsetse fly bites, reacted essentially against two saliva protein bands, those of 43–45 kDa and 28 kDa. Transcriptome analyses (Li et al., 2001; Van Den Abbeele et al., 2007) allowed the identification of Tsetse Antigen5 (TAg5, 28.9 kDa) as a putative allergen while MALDI-TOF mass spectrometry analyses confirmed the presence of TAg5 as a soluble protein in tsetse fly saliva (Haddow et al., 2002). *In silico* analyses revealed significant similarity with Antigen5 venom allergens belonging to the Pfam A PF00188 family. Structure prediction, based on homology with the vespid allergen Ves v 5, suggests a typical α–β–α sandwich protein. Production of recombinant TAg5 in *Sf*9 insect cells allowed us to explore the correlation between human IgE responses and exposure to tsetse fly bites. In addition, we have optimized an RNA interference approach for the specific silencing of tsetse fly salivary proteins, similar to a procedure described for the knock-down of an antimicrobial peptide in the fat body tissue (Hu and Aksoy, 2006). Using a single thoracic injection of 15 μg

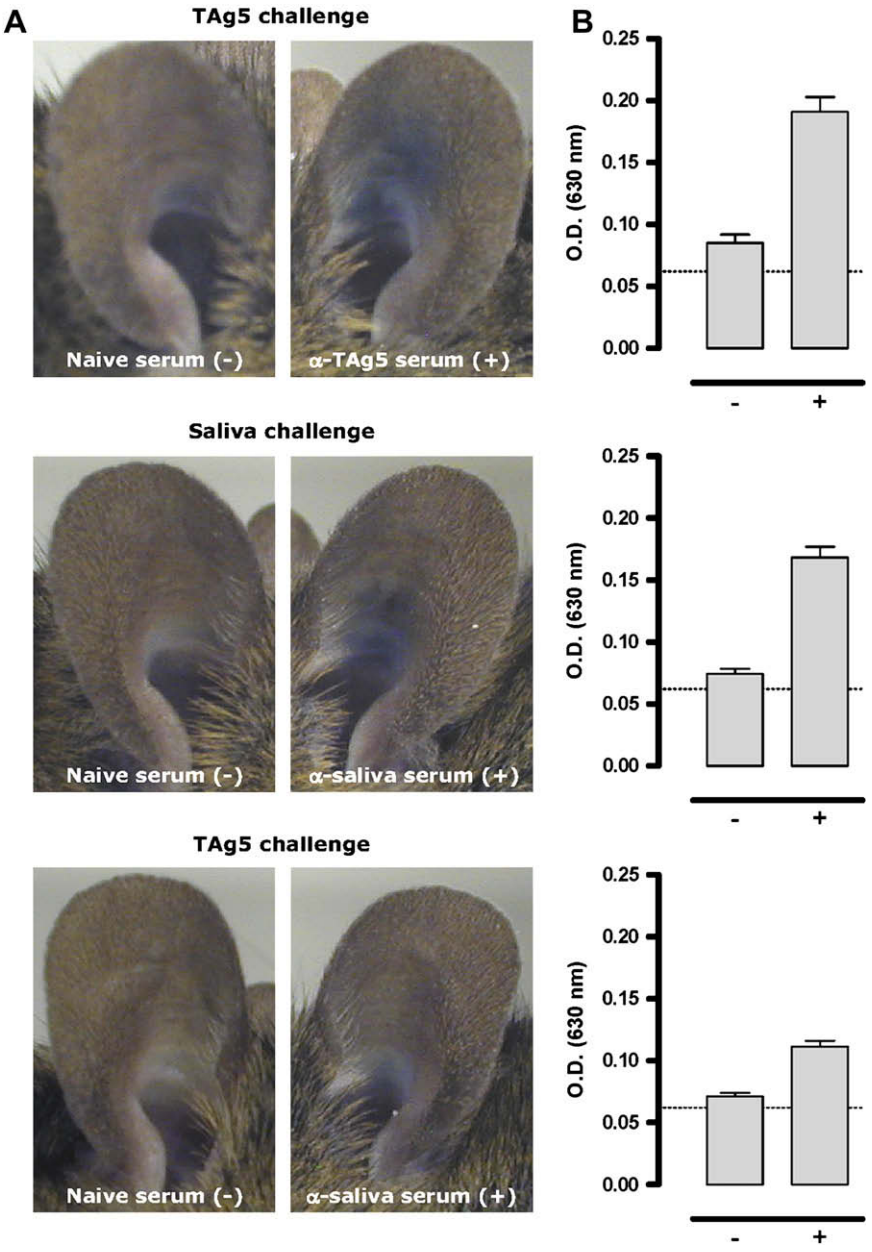

Fig. 7. Passive cutaneous anaphylaxis assay: (A) Evans blue extravasation upon intrapinna injection of ½ diluted naive (−) and immune serum (+), followed by local challenge after 24 h with TAg5 or saliva ($n = 2$/group). Pictures are those of representative responder mice. (B) Graphs represent the dye quantification by $O.D._{630nm}$ measurement in filtered formamide ear extracts.

gene-specific double stranded RNA, >90% transcriptional and >50% translational silencing could be achieved for TAg5 in the salivary gland tissue. This allowed the assessment of the relative contribution of TAg5 to the total tsetse salivary allergenicity. At first, ELISA confirmed the presence of TAg5-specific IgEs in the plasma of the anaphylaxis patient, suggesting that TAg5 might have been involved in triggering the previously reported atopical reaction (Stevens et al., 1996). By 2008, after being unexposed to tsetse bites for more than one year, this patient had undergone seroconversion as suggested by the undetectable levels of specific IgEs in his plasma. However, this does not exclude that the patient would develop hypersensitivity reactions after challenge, as insect sting anaphylaxis has been documented in patients without detectable venom-specific IgEs (Clayton et al., 1985). We next explored whether saliva and TAg5-specific IgE responses could be detected in a panel of 177 human plasmas collected in human African trypanosomiasis endemic areas of south eastern Uganda where tsetse flies (*Glossina fuscipes fuscipes*) are present (Waiswa et al., 2006). 56 and 65% of these tsetse exposed individuals scored significantly positive ($P < 0.001$) for respectively TAg5 and saliva-specific IgE responses. These responses might result from the sensitizing properties of Tsetse Antigen5 in humans although similar responses in North Senegalese plasmas suggest that sensitizing properties of Antigen5-related proteins of other insects, such as *Anopheles* mosquitoes to which the African individuals are highly exposed, result in cross-reactivity to TAg5. In non-tsetse exposed European individuals, very low saliva-reactive IgE titers were detectable and shown by specific RNAi to be TAg5 cross-reactive. This suggested that exposure to other allergens such as Antigen5 from different biting/stinging insects results in TAg5 cross-reactive

IgEs, a feature that is well established to occur to different degrees between Antigen5 representatives of hornets, wasps and yellow jackets (King et al., 1985; Hoffman, 1993; Lu et al., 1993).

Beside human TAg5-binding IgEs, western blot analyses indicated that positive responders in ELISA also recognized other tsetse saliva proteins such as the 43–45 kDa bands. This illustrated that the tsetse salivary allergome is more complex than only TAg5. Based on our RNA interference (RNAi) experiments that report for the first time on the silencing of tsetse salivary proteins, we illustrated that a significant proportion of saliva-reactive IgEs are directed against TAg5.

To further illustrate the IgE inducing potential of Tsetse Antigen5 and to demonstrate its ability to activate mast cells/basophils to release vaso-active mediators, mice were immunized against native and recombinant TAg5 followed by cutaneous hypersensitivity assays using Evans blue extravasation as read-out. Our immunization protocol was sufficient to raise specific IgEs that were associated with positive skin tests upon challenge, clearly illustrating the sensitizing and allergenic properties of TAg5. In addition, passive transfer of immune serum was able to sensitize a dermal site to respond to a subsequent allergen challenge. The murine analysis also suggested the absence of direct mast cell activators in tsetse saliva, differently as has been documented for some other blood feeding insects (Mulenga et al., 2003; Demeure et al., 2005).

Collectively, *in silico* analysis, serological analyses using recombinant TAg5 and saliva that was extracted after TAg5-specific RNAi combined with mouse allergenicity tests identified Tsetse Antigen5 as a functional salivary allergen of the Pfam A PF00188 family. As such, TAg5 could be assigned the scientific allergen name Glo m 5 as it is an allergen involved in local and possibly systemic hypersensitivity reactions. These data further indicate that Antigen5-related proteins represent a hazard in exposure to not only stinging but also hematophagous insects.

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References

Ameri, M., Wang, X., Wilkerson, M.J., Kanost, M.R., Broce, A.B., 2008. An immunoglobulin binding protein (antigen 5) of the stable fly (Diptera: Muscidae) salivary gland stimulates bovine immune responses. J. Med. Entomol. 45, 94–101.

Asojo, O.A., Goud, G., Dhar, K., Loukas, A., Zhan, B., Deumic, V., Liu, S., Borgstahl, G.E., Hotez, P.J., 2005. X-ray structure of Na-ASP-2, a pathogenesis-related-1 protein from the nematode parasite, *Necator americanus*, and a vaccine antigen for human hookworm infection. J. Mol. Biol. 346, 801–814.

Bochenek, G., Nizankowska, E., Gielicz, A., Szczeklik, A., 2008. Mast cell activation after adenosine inhalation challenge in patients with bronchial asthma. Allergy 63, 140–141.

Caljon, G., Van Den Abbeele, J., Sternberg, J.M., Coosemans, M., De Baetselier, P., Magez, S., 2006a. Tsetse fly saliva biases the immune response to Th2 and induces anti-vector antibodies that are a useful tool for exposure assessment. Int. J. Parasitol. 36, 1025–1035.

Caljon, G., Van Den Abbeele, J., Stijlemans, B., Coosemans, M., De Baetselier, P., Magez, S., 2006b. Tsetse fly saliva accelerates the onset of *Trypanosoma brucei* infection in a mouse model associated with a reduced host inflammatory response. Infect. Immun. 74, 6324–6330.

Chen, Y.L., Simons, F.E., Peng, Z., 1998. A mouse model of mosquito allergy for study of antigen-specific IgE and IgG subclass responses, lymphocyte proliferation, and IL-4 and IFN-gamma production. Int. Arch. Allergy Immunol. 116, 269–277.

Clayton, W.F., Georgitis, J.W., Reisman, R.E., 1985. Insect sting anaphylaxis in patients without detectable serum venom-specific IgE. Clin. Allergy 15, 329–333.

Demeure, C.E., Brahimi, K., Hacini, F., Marchand, F., Peronet, R., Huerre, M., St-Mezard, P., Nicolas, J.F., Brey, P., Delespesse, G., Mecheri, S., 2005. *Anopheles* mosquito bites activate cutaneous mast cells leading to a local inflammatory response and lymph node hyperplasia. J. Immunol. 174, 3932–3940.

Ellis, J.A., Shapiro, S.Z., Moi-Yoi, O.O., Moloo, S.K., 1986. Lesions and saliva-specific antibody responses in rabbits with immediate and delayed-hypersensitivity reactions to the bites of *Glossina morsitans centralis*. Vet. Pathol. 23, 661–667.

Gomes, R.B., Brodskyn, C., de Oliveira, C.I., Costa, J., Miranda, J.C., Caldas, A., Valenzuela, J.G., Barral-Netto, M., Barral, A., 2002. Seroconversion against *Lutzomyia longipalpis* saliva concurrent with the development of anti-*Leishmania chagasi* delayed-type hypersensitivity. J. Infect. Dis. 186, 1530–1534.

Haddow, J.D., Poulis, B., Haines, L.R., Gooding, R.H., Aksoy, S., Pearson, T.W., 2002. Identification of major soluble salivary gland proteins in teneral *Glossina morsitans morsitans*. Insect. Biochem. Mol. Biol. 32, 1045–1053.

Henriksen, A., King, T.P., Mirza, O., Monsalve, R.I., Meno, K., Ipsen, H., Larsen, J.N., Gajhede, M., Spangfort, M.D., 2001. Major venom allergen of yellow jackets, Ves v. 5: structural characterization of a pathogenesis-related protein superfamily. Proteins 45, 438–448.

Hoffman, D.R., 1993. Allergens in Hymenoptera venom. XXV: the amino acid sequences of antigen 5 molecules and the structural basis of antigenic cross-reactivity. J. Allergy Clin. Immunol. 92, 707–716.

Hu, C., Aksoy, S., 2006. Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans morsitans*. Mol. Microbiol. 60, 1194–1204.

Ivanciuc, O., Schein, C.H., Braun, W., 2002. Data mining of sequences and 3D structures of allergenic proteins. Bioinformatics (Oxford, England) 18, 1358–1364.

Kashino, S.S., Resende, J., Sacco, A.M., Rocha, C., Proenca, L., Carvalho, W.A., Firmino, A.A., Queiroz, R., Benavides, M., Gershwin, L.J., De Miranda Santos, I.K., 2005. *Boophilus microplus*: the pattern of bovine immunoglobulin isotype responses to high and low tick infestations. Exp. Parasitol. 110, 12–21.

Kato, H., Jochim, R.C., Lawyer, P.G., Valenzuela, J.G., 2007. Identification and characterization of a salivary adenosine deaminase from the sand fly *Phlebotomus duboscqi*, the vector of *Leishmania* major in sub-Saharan Africa. J. Exp. Biol. 210, 733–740.

King, T.P., Joslyn, A., Kochoumian, L., 1985. Antigenic cross-reactivity of venom proteins from hornets, wasps, and yellow jackets. J. Allergy Clin. Immunol. 75, 621–628.

King, T.P., Spangfort, M.D., 2000. Structure and biology of stinging insect venom allergens. Int. Arch. Allergy Immunol. 123, 99–106.

Li, S., Aksoy, S., 2000. A family of genes with growth factor and adenosine deaminase similarity are preferentially expressed in the salivary glands of *Glossina m. morsitans*. Gene 252, 83–93.

Li, S., Kwon, J., Aksoy, S., 2001. Characterization of genes expressed in the salivary glands of the tsetse fly, *Glossina morsitans morsitans*. Insect Mol. Biol. 10, 69–76.

Lu, G., Villalba, M., Coscia, M.R., Hoffman, D.R., King, T.P., 1993. Sequence analysis and antigenic cross-reactivity of a venom allergen, antigen 5, from hornets, wasps, and yellow jackets. J. Immunol. 150, 2823–2830.

Maclean, L., Odiit, M., Sternberg, J.M., 2005. Intrathecal cytokine responses in *Trypanosoma brucei rhodesiense* sleeping sickness patients. Trans. R. Soc. Trop. Med. Hyg..

Mulenga, A., Macaluso, K.R., Simser, J.A., Azad, A.F., 2003. The American dog tick, *Dermacentor variabilis*, encodes a functional histamine release factor homolog. Insect. Biochem. Mol. Biol. 33, 911–919.

Ogden, N.H., Case, A.N., Lawrie, C.H., French, N.P., Woldehiwet, Z., Carter, S.D., 2002. IgG responses to salivary gland extract of *Ixodes ricinus* ticks vary inversely with resistance in naturally exposed sheep. Med. Vet. Entomol. 16, 186–192.

Paesen, G.C., Adams, P.L., Harlos, K., Nuttall, P.A., Stuart, D.I., 1999. Tick histamine-binding proteins: isolation, cloning, and three-dimensional structure. Mol. Cell 3, 661–671.

Peng, Z., Beckett, A.N., Engler, R.J., Hoffman, D.R., Ott, N.L., Simons, F.E., 2004. Immune responses to mosquito saliva in 14 individuals with acute systemic allergic reactions to mosquito bites. J. Allergy Clin. Immunol. 114, 1189–1194.

Polman, K., Stelma, F.F., De Vlas, S.J., Sow, S., Fathers, L., Le Cessie, S., Talla, I., Deelder, A.M., Gryseels, B., 2001. Dynamics of egg counts and circulating antigen levels in a recent *Schistosoma mansoni* focus in northern Senegal. Trop. Med. Int. Health 6, 538–544.

Ribeiro, J.M., Walker, F.A., 1994. High affinity histamine-binding and antihistaminic activity of the salivary nitric oxide-carrying heme protein (nitrophorin) of *Rhodnius prolixus*. J. Exp. Med. 180, 2251–2257.

Ribeiro, J.M., Charlab, R., Valenzuela, J.G., 2001. The salivary adenosine deaminase activity of the mosquitoes *Culex quinquefasciatus* and *Aedes aegypti*. J. Exp. Biol. 204, 2001–2010.

Ribeiro, J.M., Charlab, R., Pham, V.M., Garfield, M., Valenzuela, J.G., 2004. An insight into the salivary transcriptome and proteome of the adult female mosquito *Culex pipiens quinquefasciatus*. Insect Biochem. Mol. Biol. 34, 543–563.

Schreiber, M.C., Karlo, J.C., Kovalick, G.E., 1997. A novel cDNA from *Drosophila* encoding a protein with similarity to mammalian cysteine-rich secretory proteins, wasp venom antigen 5, and plant group 1 pathogenesis-related proteins. Gene 191, 135–141.

Seitzer, U., Bussler, H., Kullmann, B., Petersen, A., Becker, W.M., Ahmed, J., 2003. Quantitative assessment of immediate cutaneous hypersensitivity in a mouse

model exhibiting an IgE response to Timothy grass allergens. Med. Sci. Monit. 9, BR407–BR412.

Stevens, W.J., Van den Abbeele, J., Bridts, C.H., 1996. Anaphylactic reaction after bites by *Glossina morsitans* (tsetse fly) in a laboratory worker. J. Allergy Clin. Immunol. 98, 700–701.

Valenzuela, J.G., Francischetti, I.M., Pham, V.M., Garfield, M.K., Ribeiro, J.M., 2003. Exploring the salivary gland transcriptome and proteome of the *Anopheles stephensi* mosquito. Insect Biochem. Mol. Biol. 33, 717–732.

Van Den Abbeele, J., Caljon, G., Dierick, J.F., Moens, L., De Ridder, K., Coosemans, M., 2007. The *Glossina morsitans* tsetse fly saliva: general characteristics and identification of novel salivary proteins. Insect Biochem. Mol. Biol. 37, 1075–1085.

Waiswa, C., Picozzi, K., Katunguka-Rwakishaya, E., Olaho-Mukani, W., Musoke, R.A., Welburn, S.C., 2006. *Glossina fuscipes fuscipes* in the trypanosomiasis endemic areas of south eastern Uganda: apparent density, trypanosome infection rates and host feeding preferences. Acta Trop. 99, 23–29.