Identification and Characterization of IgE-Binding Tropomyosins in *Aedes aegypti*

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Key Words

Aedes aegypti · IgE cross-reactivity · Mosquito allergy · Tropomyosin

Abstract

Background: The mosquito *Aedes aegypti* is a potential source of important clinically relevant allergens. However, the allergenicity and cross-reactivity of most of these has not been fully described. **Methods:** Natural wild-type mosquito tropomyosin was purified by size exclusion and anionic-exchange chromatography from an *A. aegypti* extract. Further characterization was accomplished by MALDI-TOF/TOF. Two recombinant variants of tropomyosin were obtained by expression in *Escherichia coli*. Specific IgE measurement by ELISA and skin tests for mosquito extract were performed in 12 patients with asthma or allergy rhinitis residing on the Caribbean island of Martinique. Cross-reactivity between natural *A. aegypti* tropomyosin and recombinant tropomyosins from *A. aegypti*, house dust mite, shrimp and *Ascaris lumbricoides* was analyzed by ELISA competition. **Results:** Four variants of natural tropomyosin were purified. A band of 32 kDa in SDS-PAGE representing 2 tropomyosin variants (Aed a 10.0101 and Aed a 10.0201) reacted with specific IgE

of 4 of the 12 (33%) allergic patients and with rabbit polyclonal anti-shrimp tropomyosin. A high degree of cross-reactivity (60–70%) was detected between natural mosquito tropomyosin and Blo t 10, Der p 10 and Lit v 1, and a lower degree with Asc l 3 from *A. lumbricoides* (<30%). rAed a 10.0101 inhibited IgE binding to natural *A. aegypti* tropomyosin; however, rAed a 10.0201 showed a low inhibitory capacity. **Conclusion:** Tropomyosin is a new IgE-binding protein from *A. aegypti*. Two of the 4 variants identified showed different degree of cross-reactivity with tropomyosins from other arthropods. The potential allergenic role of each variant should be further investigated.

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Introduction

Mosquitoes are insects that are distributed worldwide, and they cause IgE-mediated allergic responses in sensitized individuals [1]. Manifestations of mosquito allergy are variable and depend on the route of exposure. When the contact with the allergens occurs via skin bites (saliva allergens), the symptoms consist of mild or severe local or systemic reactions [1–4]. However, evidence suggests

that the inhalation of mosquito emanations and detritus deposited in house dust may induce allergic respiratory symptoms such as allergic asthma and rhinitis [5]. This is supported by epidemiological studies which show that 2.5–47% of individuals with asthma and/or allergic rhinitis are sensitized to mosquito allergens [6–8].

We found that sera obtained from a cohort of patients residing on the Caribbean island of Martinique suffering from allergic respiratory symptoms after the inhalation of house dust mite allergens recognize several allergens from *Aedes aegypti* [9]. We have also demonstrated cross-reactivity between *A. aegypti* and house dust mites, shrimp and cockroach extracts [10]. These findings suggest that *A. aegypti* may contain allergens that participate in the pathogenesis of respiratory allergies as inducers of a Th2 response, and the subsequent development of allergic symptoms or as a modulator of the immune response established against mites, shrimp and/or cockroaches.

Tropomyosin is a pan-allergen that belongs to a family of phylogenetically conserved proteins with multiple isoforms present in both the muscle and nonmuscle cells of vertebrates and invertebrates [11]. Allergenic tropomyosins have been described in crustaceans, molluscs, arthropods, insects and helminths [12–17]. The participation of tropomyosin in cross-reactivity between mites, cockroaches and shrimps has been demonstrated. Despite the functional and structural similarity of tropomyosins, several of their presentations are nonallergenic [18]. In some regions of tropical countries where allergies to arthropods are common, most individuals are exposed concomitantly to other tropomyosin sources, such as those from helminths. This coexposure may have clinical implications, either by modulating [19, 20] or exacerbating [21–23] a previously mounted allergic response. In these regions, *A. aegypti* and other mosquito species are highly prevalent.

The aim of this study was to purify natural wild-type *A. aegypti* tropomyosin variants and to characterize the IgE-binding capacity of these molecules. As further characterization, we cloned and expressed 2 full-length recombinant tropomyosin variants and compared their antibody-binding capacity with natural tropomyosin.

Methods

Sera

Sera were obtained from 24 (14 males and 10 females) allergic patients who attended an outpatient Allergy Clinic in Fort de France, Martinique. All patients were skin tested with a panel of common standardized inhalant allergens and with a commercially

available *Aedes communis* extract (Stallergènes, Antony, France). Patients who developed a wheal diameter >3 mm to the extracts were considered to have a positive skin prick test. Twelve of these patients had a test that was positive to mosquito extract and other arthropods (table 1). Sera were obtained after written consent was signed in the context of the study, entitled: 'Etude Epidémiologique des causes Allergiques de l'asthme en Martinique', which was funded and approved by 'Fonds de l'aide à la qualité de soins de ville' from 'la Caisse Generale de Securite Sociale de la Martinique' and the 'Comite Departemental de la Martinique contre les maladies respiratoires et la tuberculose (CDMMRT)'.

Preparation of *A. aegypti* Extract

Lyophilized and defatted *A. aegypti* raw material was purchased from GREER Labs (Lenoir, N.C., USA) and extracted 1:40 overnight (ON) with 0.1 M PBS, pH 7.2 at 4°C with constant stirring. After extraction, the mixture was centrifuged at 10,000 rpm over 10 min, the supernatant was filtered through a 0.2-µm membrane, dialyzed against distilled water in a 3,500-Da cut-off membrane (Spectra/Por Dialysis Membranes, Houston, Tex., USA) and lyophilized until usage. The protein concentration was determined by Bradford assay.

Shrimp, Mite and Nematode Recombinant Tropomyosins

Production of recombinant (r) tropomyosin from the white shrimp *Litopenaeus vannamei* (rLit v 1) was performed as follows: the nucleotide sequence of tropomyosin of *L. vannamei* (GenBank EU410072.1) was optimized for codon utilization in an *Escherichia coli* expression system, obtained by gene synthesis and cloned into the expression vector pET45b+ by GenScript (Piscataway, N.J. USA). A single colony of transformed *E. coli* BL21 was grown overnight at 37°C in LB medium with ampicillin (LBA). Afterwards, the culture was diluted 1:20 in LBA and grown at 37°C to an OD₆₀₀ of 0.5 nm. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. A cell pellet was collected by centrifugation and resuspended in denaturing binding buffer (pH 7.8) and then sonicated. His-tagged protein was purified on an Ni-NTA column under hybrid conditions (ProBond Purification System, Invitrogen), lyophilized and stored at –20°C. Production of recombinant tropomyosins from the mites *Dermaphagoides pteronyssinus* (rDer p10), *Blomia tropicalis* (rBlo t 10) and *A. lumbricoides* (rAsc l 3) was performed as previously described [17, 24].

Purification of Natural Wild-Type Tropomyosin from *A. aegypti*

Purification was performed in 2 sequential steps consisting of gel filtration and ionic-exchange high-performance liquid chromatography. In the first step, the lyophilized whole-body mosquito extract was reconstituted in 2 ml of 0.1 M PBS to a final concentration of 6 mg/ml, filtered through a 0.2-µm membrane and pre-purified by gel filtration in a HiLoad Superdex 75 prep grade, 16/600-cm column (GE Healthcare Life Sciences, Amersham, UK). The molecule of interest was eluted by applying 0.1 M PBS at a flow of 1 ml/min, and the detection was performed at a wavelength of 280 nm. Several fractions were collected and analyzed by SDS-PAGE under reducing conditions. Fractions containing the band of 32 kDa were dialyzed in a 3,500-Da cut-off membrane (Spectra/Por) against water and lyophilized for further purification by anion-exchange chromatography. The mobile phases were

Table 1. Clinical details and sensitization profiles of 12 allergic individuals used in the study

Serum	Age, years	Gender	Diagnosis	Mean wheal diameter, mm						Total IgE, IU/ml	Specific IgE against			
				<i>A. com-munis</i>	<i>D. ptero-nyssinus</i>	<i>D. farinae</i>	<i>B. tropicalis</i>	shrimp	cock-roach		<i>A. aegypti</i> extract	native tropo-myosin	rAed a 10.0101	rAed a 10.0201
1	3	M	A	6	10	6	9.5	4	0	1,260	0.379	0.052	0.062	0.077
2	8	M	R, C	4	0	0	0	0	0	626.4	0.165	0.047	0.063	0.069
3	4	M	A, C	3.5	5	3.5	4	0	0	2,187	0.458	0.386 ^a	0.289 ^a	0.158 ^a
4	9	M	A, C	4.5	5	4.5	8	3.5	3	630	0.173	0.075	0.077	0.089
5	11	M	A, R, C	4	5.5	6	5	5	3.5	2,095.4	1.679	1.683 ^a	1.417 ^a	0.991 ^a
6	5	M	A, C	3	9.5	7	3.5	0	3	324	0.319	0.052	0.078	0.076
7	13	M	R, C	6	4	3.5	3.5	0	3	2,832	0.186	0.047	0.065	0.070
8	27	F	R	5	0	0	0	0	6.5	545.5	0.355	0.179 ^a	0.480 ^a	0.182 ^a
9	15	M	A, R, C	>3	>3	>3	>3	>3	>3	1,246	1.239	0.582 ^a	0.403 ^a	0.300 ^a
10	9	M	R, C	>3	0	0	0	0	0	611	0.111	0.059	0.064	0.067
11	5	M	R, C	>3	>3	>3	>3	>3	>3	761	0.275	0.061	0.068	0.077
12	37	F	R, C	>3	0	0	0	0	0	58	0.332	0.051	0.070	0.063

A = Asthma; C = conjunctivitis; F = female; M = male; R = rhinitis.

^a Positive specific IgE.

phase A: 10 mM sodium phosphate buffer (pH 6.0) and phase B: 10 mM sodium phosphate buffer (pH 6.0) with 1.0 M NaCl. The lyophilized fraction was reconstituted in phase A and injected onto a HiPrep QFF 16/10 cm column (GE Healthcare Life Sciences). The removal of unbound molecules was carried out in a cleaning step with phase A. Bound proteins were eluted through a binary gradient of phase A and B at a flow of 3 ml/min, and the detection was performed at a wavelength of 280 nm. Fractions corresponding to the peaks that appear between 0.30 and 0.45 M NaCl were dialyzed against water and lyophilized for further analysis.

Passive Elution of Tropomyosin from Electrophoresed Gels

The fraction obtained from the HiPrep QFF column showed 4 main bands, with the 32-kDa band being the most intense. To further purify this band, the sample was separated by SDS-PAGE under reducing conditions and the band was excised from the stained gel with a clean razor. Gel pieces were placed in microcentrifuge tubes, immersed in elution buffer (50 mM Tris-HCl, 150 mM NaCl and 0.1 mM EDTA, pH 7.5), crushed using a clean pestle and then incubated in a rotary shaker at 37°C ON. The eluted protein was recovered by centrifugation at 10,000 rpm and filtration through a 0.45-μm membrane. Eluted protein was further characterized by ELISA, ELISA competition and mass spectrometry (MS).

SDS-PAGE and Immunoblotting

The mosquito extract and purified fractions were separated by SDS-PAGE under denaturing conditions using a Mini-PROTEAN II apparatus (Bio-Rad, Hercules, Calif., USA). Ten micrograms of extract or 2 μg of the purified proteins per slot were separated in 12% polyacrylamide gels at 120 volts, and the gels were stained with GelCode Blue stain reagent (Life Technologies, Carlsbad, Calif., USA). For immunoblot, proteins were electrotransferred from the polyacrylamide gel onto 0.45-μm nitrocellulose membranes using a Trans-Blot Semi-Dry electrophoresis transfer cell (Bio-Rad), at 20 v, and blocked with PBS (pH 7.4) and 0.25% Tween 20 (PBS-T), containing 5% BSA. Membranes were washed twice with PBS-

T and incubated with pooled sera (sera 3, 5, 8 and 9; table 1) from allergic individuals, diluted 1:10 in PBS-T containing 1% BSA for 2 h at 4°C. After washing with PBS-T, the membranes were incubated with 1:2,000 diluted horseradish peroxidase (HRP)-conjugated mouse anti-human IgE Fc (SouthernBiotech Birmingham, Ala., USA) for 1 h at 4°C, washed again, and then incubated with ECL prime detection reagent (GE Healthcare Life Sciences). Chemiluminescence was detected using GeneGnome apparatus (SynGene, Cambridge, UK). Otherwise, for the detection of tropomyosin, the electrotransferred and blocked membranes were incubated with 1:5,000 diluted polyclonal antibodies obtained from rabbits immunized with shrimp tropomyosin (Pen m 1; Bial Aristegui, Lisbon, Portugal), that can also bind to tropomyosins from other sources [12, 25, 26], and 1:2,000 diluted HRP-conjugated anti-rabbit IgG (Sigma-Aldrich, St. Louis, Mo., USA). Detection of bands was performed as above.

For inhibition experiments with shrimp tropomyosin, nitrocellulose strips with transferred proteins were incubated with 2 ml of pooled sera diluted 1:4 (sera 3, 5, 8 and 9; table 1) previously adsorbed ON with 10 μg/ml of Lit v 1. IgE was detected as described before.

Expression and Purification of Recombinant *A. aegypti* Tropomyosins

Production and purification of 2 tropomyosin variants was achieved as follows. The nucleotide sequence of 2 tropomyosin variants (GenBank EAT46014.1 for Aed a 10.0101 and EAT46008.1 for Aed a 10.0201) were synthesized at GenScript (Township, N.J., USA). The nucleotide sequence of Aed a 10.0201 was modified by codon optimization based on the OptimumGene™ algorithm. The synthesized nucleotide sequence of Aed a 10.0101 was subcloned into pCold IV vector (Clontech, Otsu, Japan) and the nucleotide sequence of Aed a 10.0201 was subcloned into pET-14b vector (Novagen, Billerica, Mass., USA). The vectors were used to transform *E. coli* One Shot BL21(DE3) (Life Technologies). Transformed *E. coli* was grown at 37°C in LBA with shaking at 250 rpm,

up to an OD₆₀₀ of 0.5 nm. The induction of tropomyosin expression in cells harboring pET-14b/Aed a 10.0201 plasmid was achieved by the addition of 0.5 mM IPTG and incubation for 5 h. Cells harboring pCold IV/Aed a 10.0101 plasmid were subjected to thermal shock at 15°C and incubated at this temperature during 24 h in the presence of 0.5 mM IPTG. Afterwards, the cells were harvested by centrifugation at 10,000 rpm for 10 min. Tropomyosin was partially purified by isoelectric precipitation. The cell pellet was lysed in 20 mM Tris-HCl, 1.0 M KCl, 10 mM β-mercaptoethanol (pH 7.5) and boiled at 97°C for 5 min and then centrifuged at 10,000 rpm for 5 min. The pH of the supernatant was adjusted at 4.85 with 1.0 M HCl for isoelectric precipitation, and stirred for 1 h at 4°C. The sample was centrifuged again and the precipitate dissolved in 20 mM of phosphate buffer (pH 7.4) and 0.5 M NaCl.

Purification of rAed a 10.0101 was performed following the strategy proposed by Erban [27], with some modifications. The sample containing the recombinant tropomyosin was subjected to ammonium sulfate fractionation. The protein extracted at 45% of ammonium sulfate was dialyzed against Tris-buffered saline (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl) and the sample loaded onto a HiTrap Benzamidine FF (high sub) column (GE Healthcare Life Sciences) and then washed with binding buffer (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4). Finally, 0.05 M glycine (pH 3.0) was used to elute purified protein. Fractions containing recombinant tropomyosin were pooled, dialyzed in 3,500-Da cut-off membrane (Spectra/Por) and lyophilized until usage.

Purification of rAed a 10.0201 was performed in Ni-NTA resin (Life Technologies) under native conditions, according to the manufacturer's instructions. Protein fractions were analyzed by SDS-PAGE and those containing recombinant tropomyosin were pooled, dialyzed as above and lyophilized until usage.

Enzyme-Linked Immunosorbent Assay

For specific-IgE level measurement, microplates were coated ON with 0.5 µg of extract or 0.05 µg of purified tropomyosin per well, and diluted in 100 µl of 0.05 M carbonate/bicarbonate buffer (pH 9.6). After washing 3 times with PBS-T, nonspecific binding was blocked for 1 h with PBS-T containing 1% BSA, followed by 3 more washing steps. Microplates were then incubated for 2 h with sera, diluted 1:8 in PBS-T containing 1% BSA and, after washing, incubated for 1 h with 1:2,000 diluted HRP-conjugated mouse anti-human IgE Fc (Southern Biotech). Finally, microplates were washed and incubated with *o*-phenylenediamine dihydrochloride (Sigma-Aldrich). Reaction was stopped with HCl and absorbance was read at 492 nm. All samples were assayed in duplicate. Sera from 9 subjects with pollen allergy were used as negative controls. 'No antigen' background values were subtracted from test sera data. Three standard deviations above the mean OD₄₉₂ nm value from the control subject sera were used to determine the cut-off for positive IgE binding.

ELISA Competition Assay

To study the cross-reactivity between natural *A. aegypti* tropomyosin and tropomyosin from other organisms (rDer p 10, rBlo t 10, rLit v 1 and rAsc l 3) or between natural *A. aegypti* tropomyosin and the 2 recombinant tropomyosin variants, ELISA competition assays were performed. *Betula verrucosa* extract prepared at Immunotek, SL, Madrid, Spain, was used as negative control. A pool of sera from 3 allergic patients (sera 3, 5 and 9; table 1) with IgE against purified natural *A. aegypti* tropomyosin were diluted 1:4 in

PBS-T containing 1% BSA. Microplates were coated with antigen and blocked as described above. Subsequently, 50 µl of serially diluted competitor and 50 µl of diluted sera were added to the wells. The final dilution of the sera was 1:8. Microplates were incubated ON at 4°C. The reaction was developed as described before. The pool adsorbed with PBS-T containing 1% BSA without competitor served as a positive control. Percent inhibition was calculated using the following equation: percent inhibition = 100 - [(OD of serum with competitor/OD of serum without competitor) × 100].

Proteomic Analysis

MS analysis was realized at the Hospital Nacional de Paraplégicos Proteomic Unit, Toledo, Spain, as previously described [28]. The band of 32 kDa was excised from the 1-dimensional electrophoresis gel, reduced with 10 mM dithiothreitol (Sigma-Aldrich), alkylated with 55 mM iodoacetamide (Sigma-Aldrich) and digested with sequencing-grade modified porcine trypsin (Promega, Madison, Wis., USA) at a final concentration of 12.5 ng/µl according to Shevchenko et al. [29]. Peptides were desalted on a precolumn (Acclaim PepMap 100 C18, 5 µm, 100 Å; 300 µm i.d. × 5 mm, LC Packings), followed by separation on an UltiMate™ Nano LC system (Dionex) using an Onyx Monolithic C18 column (150 × 0.1 mm, Phenomenex Inc., Torrance, Calif., USA). Fractionation of the peptides was performed with a Probot™ Microfraction Collector (Dionex). CHCA (Sigma-Aldrich) was used as a MALDI matrix. MS and tandem MS (MS/MS) analysis of offline spotted peptide samples were performed using the 4800 plus MALDI TOF/TOF Analyzer mass spectrometer (Sciex). The parent ion of Glu-1 fibrinopeptide (Sigma-Aldrich) at *m/z* 1570.677, diluted in the matrix (2 fmol per spot), was used for internal calibration. Up to 10 of the most intense ion signals per spot position, with *s/n* >40 were selected as precursor for MS/MS acquisition, excluding common trypsin autolysis peaks and matrix ion signal. Peptide and protein identifications were performed using ProteinPilot™ software v5.0 (Sciex) and the Paragon algorithm [30]. Each MS/MS spectrum was searched against a Uniprot/Swiss-Prot protein sequence database entry (October 2013), with the fixed modification of carbamidomethyl-labeled cysteine parameter enabled. Other parameters, such as the tryptic cleavage specificity, the precursor ion mass accuracy and the fragment ion mass accuracy, are MALDI 4800 built-in functions of ProteinPilot software. A search against a concatenated database containing both forward and reversed sequences enabled the false discovery rate to be kept at <1%.

Results

Purification of Tropomyosin from an *A. aegypti* Extract

The fraction collected at retention times of 40–52 min in the gel filtration contained a 32-kDa band, which bound specific IgE. When separated by ionic-exchange chromatography, this band showed a spectrum with peaks II and III at 0.30–0.45 M NaCl containing proteins of interest (fig. 1a). The immunoblotting showed that peak II corresponded to a mixture of 4 principal bands of 50, 32, 27 and 18 kDa, which also reacted with a human

Fig. 1. Purification and IgE reactivity of natural tropomyosin from *A. aegypti*. **a** Ionic-exchange chromatography spectrum. **b** Peaks II and III obtained by ionic exchange contained 5 principal bands in the SDS-PAGE (strips 1 and 5) and reacted with serum IgE from

allergic patients (strips 2 and 6) and polyclonal rabbit anti-shrimp tropomyosin (strips 3 and 7). Adsorption of human pooled sera with 10 µg/ml rLit v 1 abrogated the reactivity (strips 4 and 8).

Table 2. Proteins obtained from an *A. aegypti* extract after purification by gel electrophoresis and ionic-exchange liquid chromatography and identified by MS/MS analysis

Band No.	Protein	Uniprot code	Coverage, %	Theoretical MW, kDa	Experimental MW, kDa
1	myosin	Q178Y4	19.87	224.21	18
2	tropomyosin	Q17H76	76.75	31.001	27
3	tropomyosin	Q17H75	78.59	32.722	32
		Q17H80	78.87	32.465	32
4	myosin	Q178Y4	27.05	224.21	50
5	tropomyosin	Q17H76	52.76	31.001	32
		Q17H82	47.53	32.376	32

serum with positive IgE to *A. aegypti* and the rabbit anti-Pen m 1 polyclonal antibody (fig. 1b). The reactivity significantly decreased when both human and rabbit sera had been incubated with 10 µg of Lit v 1, suggesting that the bands contained tropomyosin, which cross-react with shrimp tropomyosin.

Characterization of Natural Tropomyosins from Purified Fractions

MS/MS analysis enabled the identification of 4 tropomyosin versions, previously reported in Uniprot (Uniprot codes Q17H75, Q17H76, Q17H80 and Q17H82) and 2 myosin products (table 2). The 32-kDa band excised from the SDS-PAGE gel corresponded to a mixture of 2

tropomyosins (Q17H75 and Q17H80) which showed IgE binding capacity. Even though these 2 molecules migrated at the same level in the gel, our MS/MS analysis was accurate enough to determine the sequence of specific peptides which enabled us to identify the presence of these 2 protein variants. Amino acid sequence alignment analysis showed 95.1% identity between Q17H75 and Q17H76, and 96.1% identity between Q17H80 and Q17H82. However, the percentage of identity between Q17H75 and Q17H80, and between Q17H82 and Q17H76, was 63% and 63.5%, respectively.

The band 3 (the mixture of Q17H75 and Q17H80 variants) showed IgE reactivity with 4 of 12 sera (33.3%) (table 1). The IUIS Allergen Nomenclature Subcommittee

Fig. 2. Amino acid sequences of 2 *A. aegypti* tropomyosins (Aed a 10.0101 and Aed a 10.0201). A sample from ionic-exchange chromatography (fig. 1b: band 3, strip 1) was analyzed by MS/MS. The peptide sequences identified are highlighted.

Q17H75 (Aed a 10.0101)				
10	20	30	40	50
MDAIKKKKQA	MKLEKDNALD	RALLCEQQAR	DANLRAEKAE	EEARQLQKKI
60	70	80	90	100
<u>QAIENDLDQT</u>	<u>QEALMAVNAK</u>	<u>LEEKEKALQN</u>	<u>AESEVAALNR</u>	<u>RIQLLEEDLE</u>
110	120	130	140	150
<u>RSEERLASAT</u>	<u>AKLSEASAAA</u>	<u>DESERARKVL</u>	<u>ENRALADEER</u>	<u>MDALENQLKE</u>
160	170	180	190	200
<u>ARFMAEEADK</u>	<u>KYDEVARKLA</u>	<u>MVEADLERAE</u>	<u>ERAEAGEGKI</u>	<u>VELEELRVV</u>
210	220	230	240	250
<u>GNNLKSLEVS</u>	<u>EEKANQREEE</u>	<u>YKNQIKTLTT</u>	<u>RLKEAEARAE</u>	<u>FAERSVQKLQ</u>
260	270	280		
KEVDRLLEDEL	VMEKEKYREI	GDDLDTAFVE	LILKE	

Q17H80 (Aed a 10.0201)				
10	20	30	40	50
MDAIKKKKQA	MKLEKDNAAD	KADTCENQAK	EANLRADKIM	EEVAELTKRL
60	70	80	90	100
<u>TQVTEDHEKF</u>	<u>KNTLEQANKD</u>	<u>LEEKEKLLTS</u>	<u>TEANVAALTR</u>	<u>KVQQVEEDLE</u>
110	120	130	140	150
<u>KSEERSGAAL</u>	<u>SKLLEATQSA</u>	<u>DENNRMCKVL</u>	<u>ENRSQQDEER</u>	<u>MDQLSNQLKE</u>
160	170	180	190	200
<u>ARMLAEDADG</u>	<u>KSDEVSRKLA</u>	<u>FVEDELEVAE</u>	<u>DRVKSGEAKI</u>	<u>MELEELKVV</u>
210	220	230	240	250
<u>GNSLKSLEVS</u>	<u>EDKANQRVEE</u>	<u>FKRQLKSLTI</u>	<u>KLKEAETRAE</u>	<u>NAEKNVKKLQ</u>
260	270	280		
KEVDRLLEDKL	MNEKDKYKAI	CDDLDTSTFAE	LTGY	

(www.allergen.org) has assigned to these tropomyosins the following official numbers: Aed a 10.0101 for Q17H75 and Aed a 10.0201 for Q17H80. These allergens share 60.2% of identity in the amino acid sequence (fig. 2).

Analysis of sequence alignments showed a greater similarity of Aed a 10.0101 with the other arthropod tropomyosins than that shown by Aed a 10.0201. The comparative analysis of amino acid sequence of the IgE-binding epitopes of tropomyosin from mite, cockroach and shrimp [31–33] also showed the greater similarity of the Aed a 10.0101 variant in these epitope regions (fig. 3). Thus, 4 putative epitopes, corresponding to residues 87–101 (ALNRRRIQLLEEDLER), 137–141 (DEERM), 144–151 (LENQLKEA) and 249–260 (LQKEVDRLLEDEL), were completely conserved in the amino acid sequence of Aed a 10.0101. The epitope ESKIVELEEL (residues 187–197) is modified by a single change EGKIVELEEL in Aed a 10.0101. Only 1 epitope, corresponding to residues 137–141, was conserved in Aed a 10.0201 (fig. 3).

Tropomyosin from A. aegypti Cross-React with the Other Allergenic Tropomyosins

ELISA competition assays showed that when *A. aegypti* tropomyosin was in the solid phase, homologous inhibition was 61.48% at 2 µg/ml of competitor. At this concentration, rBlo t 10, rDer p 10 and rLit v1 reached 75, 73.98 and 61.54% of inhibition respectively, whereas rAsc l 3 reached only 23.68% (fig. 4).

Recombinant Aed a 10.0101 and Aed a 10.0201 Tropomyosins Showed Different IgE-Binding Capacities

On SDS-PAGE, rAed a 10.0101 migrated with an apparent molecular weight (MW) of 32 kDa and rAed a 10.0201 migrated at 34 kDa. Both molecules exhibited IgE- and IgG-binding capacities when incubated with human sera and rabbit anti-shrimp tropomyosin antibody, respectively (fig. 5). However, bands with a higher MW that may correspond to dimers or trimers were also ob-

served in the immunoblotting. Cross-inhibition by IgE competition ELISA between the natural purified tropomyosin mixture and both recombinant variants showed that Aed a 10.0101, or an equimolar mixture of Aed a

10.0101 and Aed a 10.0201, were able to abrogate all the IgE binding to the natural tropomyosin mixture. In contrast, competition with Aed a 10.0201 only inhibited the IgE reactivity to natural tropomyosin up to 30% (fig. 6).

Fig. 3. Multiple-sequence alignment between *A. aegypti* tropomyosins (Aed a 10.0101 and Aed a 10.0201) and other tropomyosins. IgE-binding epitopes of Pen m 1 are indicated in boxes (described by Ayuso et al. [32]). Conserved epitopes in Aed a 10.0101 and Aed a 10.0201 are highlighted in red. Differences in the amino acid sequences of Der p 10, Blo t 10 and Asc l 3 are indicated in bold type.

Discussion

In this work, we report that tropomyosin from *A. aegypti* has IgE reactivity and its natural source is represented for at least 4 variants, 2 of them more related to tropomyosin from mites and shrimp than to that from *Ascaris lumbricoides*. Although >3,500 species of mosquito have been described, we have focused our work on *A. aegypti* because this mosquito has experienced a rapid global spread and is commonly found in America, central and southern Africa, Oceania, Asia and, to a lesser extent, in Europe [34], where it acts as a vector of several pathogens [35, 36]. Furthermore, *A. aegypti* has evolved to cohabit and feed from humans [37] who may be constantly exposed to the mosquito antigens.

Using a combination of 2 chromatographic techniques and proteomic analysis, 4 tropomyosin variants were identified in this study. These variants coeluted with truncated myosin on the ion-exchange column, which can be explained by the common association of

Fig. 4. IgE competition analysis. Microplate wells coated with natural *A. aegypti* tropomyosin were incubated with human pooled sera and different concentrations of rBlo t 10, rDer p 10, rLit v 1, rAsc l 3, natural *A. aegypti* tropomyosin or *B. verrucosa* extract.

Fig. 5. a Gel electrophoresis and Coomassie staining of rAed a 10.0101 and rAed a 10.0201. Immunoblots of these recombinants incubated with pooled sera from allergic patients (b) or rabbit polyclonal anti-shrimp tropomyosin (c).

Fig. 6. IgE competition ELISA results. Microplate wells coated with natural *A. aegypti* tropomyosin were incubated with human pooled sera and different concentrations of rAed a 10.0101, rAed a 10.0201 or an equimolar mixture of these recombinants. Natural *A. aegypti* tropomyosin for homologous inhibition and *B. verrucosa* extract as unrelated antigens were used. At the highest concentration of the competitor rAed a 10.0201, 30% IgE-binding inhibition was obtained.

these proteins to the thin filament and microfilaments, together with actin, in all eukaryotic cells [11]. A band of 32 kDa was identified as a mixture of 2 variants, Aed a 10.0101 and Aed a 10.0201. These molecules have similar physicochemical characteristics, inducing them to eluting at the same retention time in gel-filtration and ion-exchange chromatographic techniques. The mixture of the natural tropomyosins reacted with the IgE in 33% of the allergic individuals. Further experiments should be performed in order to clarify the contribution of each variant in the pattern of reactivity observed. The higher degree of cross-reactivity between the natural mosquito tropomyosin and tropomyosin from mites and shrimp is consistent with the amino acid sequence analysis, suggesting the presence of potential IgE cross-reacting epitopes as well as with the close taxonomic relationship across these species.

The cross-reactivity study allowed us to hypothesize that mite tropomyosin is probably the primary source of sensitization, which means that sensitization to mosquito tropomyosins is influenced by sensitization to mite

allergens. This aspect may have important clinical implications, i.e. the diagnosis of arthropod-induced allergy may be biased by cosensitization to *A. aegypti*, and this mosquito species may represent a source of IgE-cross-reactive molecules and not be a genuine sensitizing molecule. On the other hand, it is unknown if exposure to *A. aegypti* tropomyosin could have a role in the regulation of immune response induced by mite and shrimp tropomyosins.

In Africa and South America, the prevalence of sensitization to mite tropomyosin is approximately 50% [38, 39], higher than those observed in developed countries [40, 41]. These differences are partially explained by the presence of other Th2-inducing and cross-reactive antigens, such as *Ascaris* [17, 23]. Our results of specific IgE-binding and cross-reactivity suggest that *A. aegypti* tropomyosin may play a role in the sensitization pattern of allergic populations residing in areas where mosquitoes are highly prevalent.

The alignment of amino acid sequences of Aed a 10.0101 and Aed a 10.0201 with those from other arthropods suggests the presence of putative IgE epitopes, which are not yet experimentally verified. Aed a 10.0101 showed a higher sequence identity with tropomyosins from other allergenic sources when compared to Aed a 10.0201 (fig. 3), including the regions of putative IgE epitopes. Based on these observations, we hypothesized that Aed a 10.0101 could have a higher IgE reactivity (supported by the ELISA results with the 12 sera showing that the 4 positive IgE sera had higher levels of antibodies to this variant) than Aed a 10.0201 (table 1). These results suggest that Aed a 10.0101 is more represented in the mosquito proteome. Further studies should be performed to evaluate the potential this protein may have for the diagnosis of allergy caused by arthropods, since it seems to have a wide representation of IgE epitopes related to tropomyosin.

In conclusion, we describe that the whole-body extract of *A. aegypti* contains 2 variants of tropomyosin which can mediate IgE cross-reactivity with allergenic tropomyosins from house dust mites, shrimp and the nematode, *A. lumbricoides*, at different levels. The recombinant molecules of these variants showed different IgE-binding properties. The analysis of the immune response elicited by mosquito tropomyosin and other mosquito antigens and the establishment of whether or not these molecules are present in house dust, or the outdoor environment will help to clarify the allergenic role of *A. aegypti* and to design better schemes for the diagnosis and treatment of mosquito-induced allergies.

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Disclosure Statement

The authors report no conflict of interests in relation to this paper.

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