

Cloning and Characterisation of Two IgE-Binding Proteins, Homologous to Tropomyosin and α -Tubulin, from the Mite *Lepidoglyphus destructor*

Tiiu Saarne Liselotte Kaiser Omid Rasool Sonia Huecas
Marianne van Hage-Hamsten Guro Gafvelin

Department of Medicine, Unit of Clinical Immunology and Allergy, Karolinska Institutet and Hospital, Stockholm, Sweden

Key Words

Mite allergy · *Lepidoglyphus destructor* · Lep d 10 · Tropomyosin · α -Tubulin · Recombinant allergens

Abstract

Background: The dust mite *Lepidoglyphus destructor* is a major source of mite allergy in European rural environments, but it also causes allergy in urban populations around the world. We have previously cloned, sequenced and expressed several allergens from *L. destructor* (Lep d 2, Lep d 5, Lep d 7 and Lep d 13). The aim of this study was to identify and clone additional allergens from *L. destructor*, and to evaluate their IgE-binding reactivities. **Methods:** PCR and screening with sera from *L. destructor*-sensitised individuals were used to isolate new clones from a phage display *L. destructor* cDNA library. The complete coding sequences of the clones were determined and expressed as His₆-tagged recombinant proteins in *Escherichia coli*. The recombinant proteins were analysed by SDS-PAGE, immunoblotting and mass spectrometry. **Results:** Two new clones, showing homology to tropomyosin and α -tubulin in several species, were isolated from the phage display *L. destructor* cDNA library. Due to its homology to group 10 dust mite allergens, the tropomyosin clone was named Lep d 10.

The IgE-binding frequencies of the recombinant Lep d 10 and α -tubulin were 13% (18/136) and 12% (11/95), respectively, among subjects with IgE reactivity to mites and/or crustaceans. **Conclusions:** Two new allergens from *L. destructor* have been identified and can now be added to the repertoire of recombinant *L. destructor* allergens. In addition, both these allergens belong to highly conserved protein families and may be important for evaluation of allergenic cross-reactivity.

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Introduction

Many species of dust mites have been found in European agricultural environments [1, 2] and several studies have shown that occupational exposure to dust mites causes allergic disease in farmers [3], grain handlers [4] and bakers [5]. In the last decade, sensitisation to these mite species has also been demonstrated in different urban populations [6, 7]. One of the most common species that causes mite allergy in the rural environment in Europe, is *Lepidoglyphus destructor* [2].

By analysing crude extract of *L. destructor* by SDS-PAGE and immunoblotting, Johansson et al. [8] identified 21 IgE-binding components. The first allergen from

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Correspondence to: Tiiu Saarne
Karolinska Institutet, Department of Medicine
Unit of Clinical Immunology and Allergy, Karolinska Hospital, L2:04
SE-171 76 Stockholm (Sweden)
Tel. +46 8 51776697, Fax +46 8 335724, E-Mail tiiu.saarne@ks.se

Table 1. Primers used for PCR, RACE-PCR and sequencing

	Sequence
<i>Forward primers</i>	
T7 promoter	5'-TAATACGACTCACTATAGGG-3'
Trop1	5'-CATATGGANGCCATCAARAANAARATG-3'
Trop2	5'-GAGCATATGGAGGCCATCAAAAATAAGATGCAGGCT-3'
Trop3	5'-GAGGGCTTGGAAAAGTCAGCTC-3'
Tub1	5'-CACCATATGCGTGAATGTATCTCAGTC-3'
Tub2	5'-CCGTAGTTGATGAAGTTCGTA-3'
Tub3	5'-GAGCCATACAACAGTATTCTTA-3'
Tub4	5'-GTCACCTATGCCCCAGTTAT-3'
Tub5	5'-ACTGTTGTTCCAGGTGGTGATCT-3'
<i>Reverse primers</i>	
Trop4	5'-ATCGGCTTCAACCATTGCCAATTT-3'
Trop5	5'-GGTAACTTAATAACCAGTNAGYTCNGCRAA-3'
Trop6	5'-GAGGGATCCTTAATGATGGTGGTGGTGGTGATAACCAGTAAGTTCAGC-3'
Tub6	5'-TACGAACCTCATCAACTACGG-3'
Tub7	5'-CACCACGGTACAACAAACAACAG-3'
Tub8	5'-GCTTCGGAGAATTCACCTTCTT-3'
Tub9	5'-GAGGGATCCTTAGTGATGGTGGTGGTGATGAAATTCTTCTCCAGCGGTATC-3'
T7 terminator	5'-GCTAGTTATTGCTCAGCGG-3'

L. destructor to be cloned [9], sequenced [9, 10] and expressed as a recombinant protein [11] was the major allergen Lep d 2. The IgE-binding frequency of recombinant (r) Lep d 2 among *L. destructor*-sensitised patients was shown to be 73% in a Pharmacia CAP RAST® assay [12], and in a skin prick test, the allergen was detected by 59% of mite-sensitised subjects [13]. Recently, we constructed a phage display cDNA library from *L. destructor* in order to identify and clone additional allergens from this mite species [14]. Three IgE-binding proteins, Ld 5 (originating from a partial Lep d 5 clone), Lep d 7 and Lep d 13, were isolated from the cDNA library, sequenced, expressed as recombinant proteins and shown to be new *L. destructor* allergens [14]. The rLd 5 was recognised by 9%, rLep d 7 by 62% and rLep d 13 by 13% of patients ImmunoCAP-positive to *L. destructor* extract. Thus, only 4 of the 21 potential IgE-binding components identified in *L. destructor* extract [8] have been cloned and characterised as allergens so far. The aim of this study was to find new allergens in *L. destructor*, to produce them as recombinant proteins and characterise their IgE-binding reactivity in order to increase the panel of recombinant allergens that may be used in component-resolved diagnostics [15, 16] in the future.

Strong cross-reactivity has been found between allergens from many invertebrate sources. The cross-reactivity is believed to be caused by highly conserved proteins [17].

One such group of proteins is tropomyosins, which form coiled-coil dimers in muscle and non-muscle cells of both invertebrates and vertebrates [18]. Tropomyosin has been identified as an allergen in several invertebrate species [17], including the mite species *Dermatophagoides farinae* [19], *Dermatophagoides pteronyssinus* [20] and *Blomia tropicalis* [21]. Another structural protein that is present in all eukaryotic cells is α -tubulin, which together with β -tubulin forms tubulin heterodimers, the major building blocks of microtubuli in the cytoskeleton [22].

In this study we have identified *L. destructor* tropomyosin and α -tubulin as two potential allergens with putative high cross-reactivity.

Materials and Methods

Construction of Phage Display cDNA Library and Biopanning

A phage display cDNA library, based on mRNA isolated from *L. destructor*, was constructed previously in our laboratory [14] by using the pJuFo vector [23]. Isolation, analysis and selection of phage displaying IgE-binding proteins (biopanning) were performed as described earlier by Eriksson et al. [14]. The biopanning procedure was repeated five times.

PCR Amplification

The primers used in the PCR amplifications are shown in table 1. The oligonucleotides were synthesised by Cybergene AB (Huddinge, Sweden) and DNA Technology A/S (Aarhus, Denmark). The oligo-

nucleotide primers Trop1 and Trop5, which were constructed on the basis of conserved 5'- and 3'-regions of the allergens Der f 10 and Der p 10 from *D. farinae* and *D. pteronyssinus*, respectively, were used to amplify tropomyosin-encoding DNA from the phage display *L. destructor* cDNA library. The PCR reaction was carried out in Advantage 2 PCR buffer (Clontech Laboratories Inc., Palo Alto, Calif., USA), containing cDNA, 5'- and 3'-primers at 0.4 μ M, 0.2 mM dNTPs and Advantage 2 polymerase mix (Clontech). The PCR was carried out for 30 cycles: 30 s at 95°C, 1 min at 50°C and 2 min at 68°C.

In order to obtain the full-length sequence of the α -tubulin-homologous clone, 5'- and 3'-RACE-PCR were performed with the primers Tub7 and Tub5 (table 1), respectively, on adaptor-ligated *L. destructor* cDNA. The Marathon™ cDNA Amplification Kit (Clontech) and the SMART™ RACE cDNA Amplification Kit (Clontech) were used, according to the manufacturer's protocols.

The complete coding sequences for *L. destructor* tropomyosin and α -tubulin were amplified, in order to be cloned into expression vectors, by using gene-specific primers (table 1). Tropomyosin was amplified from the phage display *L. destructor* cDNA library, using the primers Trop2 and Trop6. α -tubulin was amplified from total cDNA of *L. destructor*, using the primers Tub1 and Tub9. The primers Trop2 and Tub1 contain an *Nde* I restriction site, including a start codon, at the 5'-end of the respective coding sequences of *L. destructor* tropomyosin and α -tubulin. The primers Trop6 and Tub9 contain the 3'-ends followed by six histidine codons, a stop codon and a *Bam*HI restriction site. Tropomyosin was amplified under the same PCR conditions as described above. The PCR for α -tubulin amplification was carried out in *Pfu* polymerase buffer (Stratagene, La Jolla, Calif., USA), containing cDNA, 5'- and 3'-primers at 0.2 μ M, 0.2 mM dNTPs and 2.5 U *Pfu* polymerase (Stratagene). Thirty cycles of PCR were performed: 30 s at 94°C, 30 s at 60°C and 1 min at 72°C.

Subcloning and DNA Sequencing

Purified PCR products were either subcloned into pT7Blue blunt vector (for tropomyosin clone), by using Perfectly Blunt™ Cloning Kit (Novagen Inc., Madison, Wisc., USA), or into pCR®4-TOPO® vector (for α -tubulin clone), by using TOPO TA Cloning® Kit (Invitrogen, Groningen, The Netherlands). *Escherichia coli* XL2-Blue cells (Novagen) or One Shoot® TOP10 *E. coli* cells (Invitrogen) were then transformed by the plasmids including tropomyosin and α -tubulin, respectively. Clones with correct inserts were confirmed by restriction analysis and by DNA sequencing. The DNA sequencing was performed on an ABI Prism™ 377 DNA Sequencer (Perkin Elmer, Foster City, Calif., USA), using the ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) and the ABI Prism™ BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Perkin Elmer), according to the manufacturer's recommendations. All oligonucleotide primers used for sequencing are listed in table 1. The sequences were analysed using NCBI non-redundant SWISS-PROT protein database.

Sequences corresponding to the correct ORF of each cDNA were cleaved with the restriction enzymes *Nde* I and *Bam*HI (New England Biolabs Inc., Beverly, Mass., USA), and ligated into the expression vector pET17b (Novagen). Non-expressing *E. coli*, Nova-Blue Singles™ competent cells (Novagen) or One Shoot® TOP10 cells (Invitrogen), were transformed by the ligation reactions for sequence confirmation.

Expression and Purification of Recombinant Proteins

The *E. coli* expression strains BL21(DE3)pLysS (Novagen) and BL21-CodonPlus(DE3)-RP (Stratagene) were transformed by correct ligation constructs for tropomyosin and α -tubulin, respectively. After induction of the transformed BL21 cells, performed according to the Novagen pET System Manual, the recombinant proteins were found in two different fractions of the respective expressing cells. Recombinant tropomyosin was recovered in the cytoplasmic fraction of the cell lysate. FPLC ion exchange chromatography on a High Q 1 ml column (Bio-Rad Laboratories, Hercules, Calif., USA) was performed on this fraction with 20 mM Tris-HCl pH 8 and a salt gradient of 0–0.5 M NaCl. Recombinant tropomyosin was eluted at approximately 0.25 M NaCl and was further purified by immobilised metal chelate affinity chromatography (IMAC) using Talon® Metal Affinity Resin (Clontech). The protein was eluted from the affinity resin in 20 mM Tris-HCl pH 8, 100 mM NaCl, containing 100 mM imidazole, and then dialysed against PBS. Before disruption of the α -tubulin-expressing cells, Complete™, Mini protease inhibitor cocktail tablets (Boehringer Mannheim GmbH, Mannheim, Germany) were added to the cell suspension. The recombinant α -tubulin was found in insoluble inclusion bodies, which were solubilised in 20 mM Tris-HCl, pH 8, 100 mM NaCl, 6 M guanidine-HCl. The protein was further purified by IMAC, as described above, with the following exceptions. The protein was bound to the resin in the presence of the denaturing solubilisation buffer and the resin-bound α -tubulin was washed with a gradient of 6 to 0 M guanidine-HCl before elution with the imidazole-containing buffer. The purity of the isolated recombinant proteins was analysed on 12% SDS-PAGE gels, which were run under reducing conditions and stained with Coomassie Brilliant Blue R (Sigma-Aldrich Inc., Saint Louis, Mo., USA).

Quadrupole Time-of-Flight Electrospray Ionisation Mass Spectrometry (Q-TOF MS)

The purified recombinant proteins were analysed using nano-electrospray mass spectrometry with a Q-ToF™ instrument (Micromass, Manchester, UK) operated according to the manufacturer's recommendations. Calibration was carried out using horse heart myoglobin (16,951.5 D). The mass spectra were recorded via scanning from *m/z* 35–3,000 for 3.5 min with the needle set at 1.3–1.5 kV. The *m/z*-data were transformed into a true mass scale using software supplied by Micromass.

Preparation of Mite Extract and Purification of Native Tropomyosin from *L. destructor*

For immunoblotting and blotting inhibition experiments, extract was prepared from whole *L. destructor* culture from Allergon AB (Ängelholm, Sweden), as previously described [24]. Starting material for purification of native *L. destructor* tropomyosin was prepared from the same raw material by extraction with 1 M KCl, 0.5 mM DTT, pH 7, at room temperature overnight. A protocol for purification of tropomyosin was adapted from Smillie [18] and Aki et al. [19]. In brief, after centrifugation of the extract at 5,000 g, 15 min, the supernatant was pH-adjusted to 4.6 and stirred for 30 min. The precipitate was collected by centrifugation (5,000 g, 15 min), dissolved in the extraction buffer, and adjusted to pH 7. Tropomyosin was further enriched by repeating the precipitation at the isoelectric point, pH 4.6, twice. The final precipitate was dissolved in 0.5 mM DTT, and proteins were collected by precipitation in 65% saturated ammonium sulphate.

Table 2. Characteristics of sera

	<i>Ld</i>	<i>Dp</i>	Crust.	<i>Ld</i> and <i>Dp</i>	<i>Ld</i> and crust.	<i>Dp</i> and crust.	<i>Ld, Dp</i> and crust.	Total
Tropomyosin	22	26	20	47	1	7	13	136
α -Tubulin	4	26	21	25	2	7	10	95

Number of subjects with positive ImmunoCAP values for the respective allergen sources used in immunoblot analysis of *L. destructor* tropomyosin and α -tubulin. *Ld* = *L. destructor*; *Dp* = *D. pteronyssinus*, crust. = crustaceans (including shrimp, crab, lobster and/or crayfish).

Serum Samples

Sera from four allergic patients with positive ImmunoCAP values (13.9–92.5 kU · litres⁻¹) to *L. destructor* were used individually in the biopanning procedure performed earlier [14]. Of 141 serum samples, ImmunoCAP-positive to *L. destructor*, *D. pteronyssinus* and/or crustaceans, 136 were used for immunodetection of tropomyosin and 95 for immunodetection of α -tubulin. The characteristics of the sera used are given in table 2. Two individual atopic sera, ImmunoCAP-negative to *L. destructor*, *D. pteronyssinus* and shrimp (*Pandalus borealis*), were used as negative controls in the immunoblot experiments.

Monoclonal Antibodies

An anti-Der p 10 monoclonal antibody (mAb), 1A6 [25], kindly supplied by Prof. R.C. Aalberse, was used in dilution 1:10,000 to detect the recombinant *L. destructor* tropomyosin. The anti- α -tubulin mAb, DM 1A (Sigma-Aldrich), was used in dilution 1:5,000,000 for detection of the recombinant *L. destructor* α -tubulin.

Immunoblotting

The recombinant proteins were subjected to 12% SDS-PAGE (0.4 µg protein per mm gel) under non-reducing (tropomyosin) or reducing conditions (α -tubulin), and electroblotted on Immobilon™-P polyvinylidene fluoride (PVDF) transfer membrane (Millipore, Bedford, Mass., USA). Also *L. destructor* whole extract (15 µg protein per mm gel) and native *L. destructor* tropomyosin were subjected to SDS-PAGE under non-reducing conditions and electroblotted on PVDF membranes. The amount of native *L. destructor* tropomyosin applied to the gel was not determined since it was under the detection limit, as measured by spectrophotometry (OD₂₈₀). The electroblotting was performed as described earlier [24], with the exception that the PVDF membrane with α -tubulin was blocked with 5% fat-free dried milk in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Triton-X-100. Immunodetection with human sera was performed as previously described [24]. The same protocol was used for immunodetection with mAbs, except that affinity-purified alkaline phosphatase-conjugated rabbit anti-mouse IgG (diluted 1:5,000 for tropomyosin and 1:2,000 for α -tubulin; Dako, Glostrup, Denmark) was used as the detecting antibody.

Immunoblot Inhibition

Homologous blotting inhibition was performed with both recombinant proteins. Individual patient sera were preincubated for 1 h at room temperature with recombinant *L. destructor* tropomyosin (20 µg/ml) or recombinant *L. destructor* α -tubulin (2, 20, 200 or 700 µg/ml). The sera were then incubated with the respective recom-

binant proteins blotted on PVDF membrane. Immunoblot inhibition was performed in the same way with purified native *L. destructor* tropomyosin on the PVDF membrane, using the same serum as for recombinant tropomyosin. For α -tubulin, inhibition of binding to the native protein was performed with *L. destructor* whole extract on the PVDF membrane after preincubation with recombinant *L. destructor* α -tubulin (100 or 200 µg/ml). Preincubation was also done with *L. destructor* whole extract (2 mg/ml) before incubation with recombinant α -tubulin blotted on PVDF membrane. PVDF strips were evaluated by densitometry scanning using a Bio-Rad Model GS-670 Imaging Densitometer with Molecular Analyst® Software version 2.1 (Bio-Rad). The inhibition was calculated using the formula: % inhibition = 100 – [100 × (X – B)/(A – B)] where X is the absorbance value obtained for inhibited protein bands on PVDF strips. The absorbance value for protein bands on PVDF strips that were incubated with serum only (without inhibitor) was taken as 100% binding of IgE (A) and B is the background value (0% IgE-binding).

Results

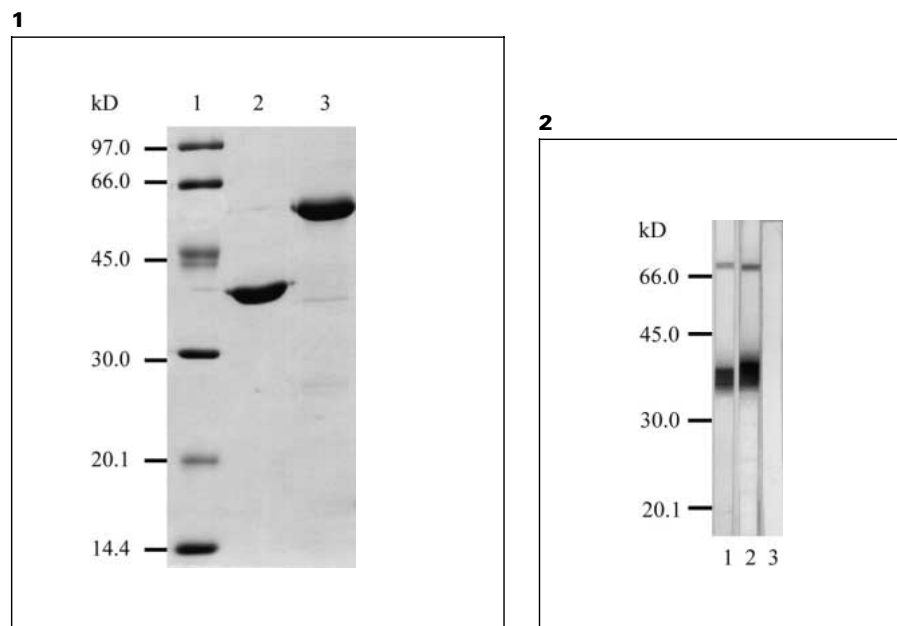
Isolation and Analysis of cDNA Clones

PCR amplification from the phage display *L. destructor* cDNA library with primers corresponding to the conserved 5'- and 3'-regions of the tropomyosins Der f 10 and Der p 10 resulted in a sequence of 852 bp, including 27 and 18 bp from the respective primer sequences. The cDNA sequence encoded 284 amino acids with a calculated molecular mass of 32,930 D. The amino acid sequence contained the two tropomyosin-like motifs EAIKNK (F-actin-binding motif) at the N-terminus, and LKEAEXRAE in the C-terminal part. No N-glycosylation sites were found in the sequence. Based on a high amino acid sequence homology (96% identity) to both Der f 10 and Der p 10 and in accordance with the WHO/IUIS Allergen Nomenclature Sub-Committee, the *L. destructor* tropomyosin was named Lep d 10 (EMBL database, accession No. AJ250096).

After five rounds of biopanning of the phage display *L. destructor* cDNA library, sequence analysis of the enriched clones revealed a clone with a partial nucleotide

Fig. 1. SDS-PAGE analysis of purified recombinant proteins. Lane 1: molecular weight marker; lane 2: rLep d 10; lane 3: recombinant *L. destructor* α -tubulin. The proteins were separated by SDS-PAGE (12% gel) under reducing conditions and stained with Coomassie Brilliant Blue R. The amount of each protein loaded was 1 μ g/mm gel.

Fig. 2. Immunoblot analysis of rLep d 10. Lane 1: detection with the anti-Der p 10 mAb 1A6; lane 2: IgE-binding with serum ImmunoCAP-positive to *L. destructor*, *D. pteronyssinus* and shrimp; lane 3: control serum ImmunoCAP-negative to *L. destructor*, *D. pteronyssinus* and shrimp.



sequence of 370 bp, showing homology (up to 86% identity) to α -tubulin of many organisms. To obtain the full-length cDNA sequence of this clone, RACE-PCR and sequencing were carried out using primers shown in table 1. Subsequently, the two primers Tub1 and Tub9, were designed to amplify and sequence the complete encoding cDNA sequence, spanning 1,350 bp, giving an ORF of 450 amino acids, and corresponding to a calculated molecular mass of 50,007 D (EMBL database, accession No. AJ428050). The amino acid sequence showed up to 94% identity to α -tubulins from other organisms, and contained the tubulin pattern S/A/G-GGTG-S/A-G at position 142–148, as well as one N-glycosylation site at position 380–382.

Production of Recombinant Proteins

The cDNAs encoding Lep d 10 and *L. destructor* α -tubulin were expressed as His₆-tagged proteins in *E. coli*. After purification, the recombinant proteins were subjected to SDS-PAGE and Coomassie staining, showing pure proteins with molecular masses of 40 kD (rLep d 10) and 50 kD (recombinant α -tubulin), which are comparable to their respective calculated molecular masses (fig. 1). As determined by mass spectrometry, the molecular masses of the His₆-tagged Lep d 10 and α -tubulin were 33,775 and 50,850 D, respectively, as compared to their calculated molecular masses of 33,752 and 50,829 D.

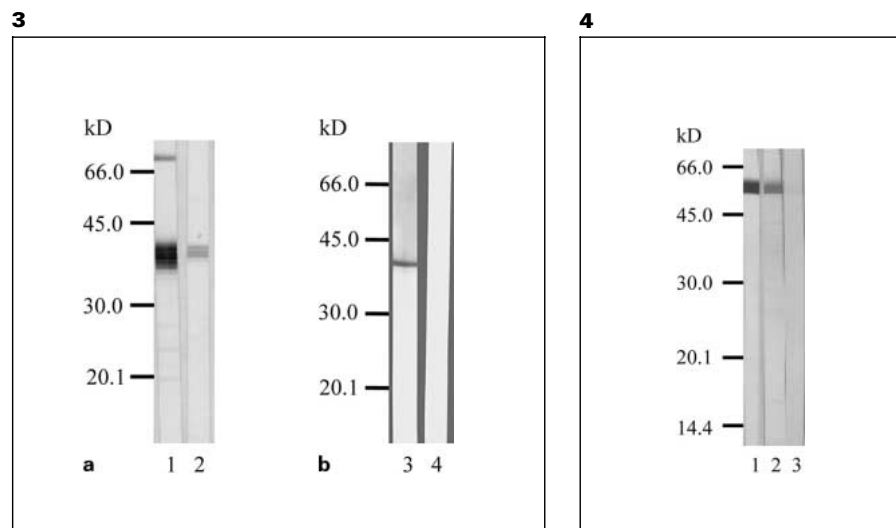
IgE Reactivity and Inhibition Analysis

Lep d 10. Purified rLep d 10 was subjected to immunoblotting experiments. A monoclonal anti-Der p 10 antibody, 1A6, recognised rLep d 10 (fig. 2), as expected from the high sequence homology between Lep d 10 and Der p 10. The IgE-binding frequency of rLep d 10 was 13% (18/136) among subjects ImmunoCAP-positive to mites and/or crustaceans. An example of an immunoblot with serum recognising rLep d 10 is shown in figure 2. All of the sera that detected rLep d 10 were ImmunoCAP-positive to shrimp (*P. borealis*), 13 were ImmunoCAP-positive to *D. pteronyssinus* and 7 were ImmunoCAP-positive to *L. destructor*. The recombinant protein was not recognised by the control sera, which lacked IgE antibodies to mites and shrimp (fig. 2). Twenty-five *L. destructor*-positive sera were also used in immunoblotting analysis with purified native (n) Lep d 10. A band of approximately 40 kD was recognised by 3 (12%) of these 25 sera (data not shown). Identical results were obtained for these 25 sera when analysed for IgE-binding to rLep d 10.

The rLep d 10 gave 93% homologous inhibition of serum IgE-binding to rLep d 10 on the PVDF membrane (fig. 3a), whereas the IgE-binding to nLep d 10 on the membrane was completely inhibited by preincubating the serum with the rLep d 10 (fig. 3b). This could be explained by the fact that the amount of rLep d 10 on the membrane was higher than that of nLep d 10, which was below the detection limit when measured by spectrophotometry.

Fig. 3. Inhibition of IgE-binding to Lep d 10. Serum ImmunoCAP-positive to *L. destructor* incubated without inhibitor (lanes 1 and 3) and with 10 µg inhibitor (lanes 2 and 4). **a** Homologous inhibition of IgE-binding to membrane-bound rLep d 10 with rLep d 10 as inhibitor. **b** Heterologous inhibition of IgE-binding to nLep d 10 on membrane with rLep d 10 as inhibitor. Note that different amounts of nLep d 10 and rLep d 10 were bound to the membranes.

Fig. 4. Immunoblot analysis of recombinant *L. destructor* α -tubulin. Lane 1: detection with the anti- α -tubulin mAb DM 1A; lane 2: IgE-binding with serum ImmunoCAP-positive to *L. destructor* and *D. pteronyssinus*; lane 3: control serum ImmunoCAP-negative to *L. destructor* and *D. pteronyssinus*.



α -Tubulin. In immunoblotting analysis of the recombinant α -tubulin, the anti- α -tubulin mAb, DM 1A, recognised a 50-kD protein (fig. 4). Immunoblotting with human sera showed an IgE-binding frequency of 12% (11/95) to recombinant α -tubulin among subjects ImmunoCAP-positive to mites and/or crustaceans (example shown in fig. 4). The IgE-binding to recombinant α -tubulin on the PVDF-membrane could not be inhibited by recombinant α -tubulin or by *L. destructor* whole extract (data not shown). α -Tubulin was very weakly detected in the *L. destructor* whole extract blotted on membrane, probably due to a very low concentration of this protein. Hence, it was not possible to interpret the results from the inhibition experiment with membrane-bound extract.

Discussion

In this study, we describe the identification, cloning, expression and characterisation of two IgE-binding components from the dust mite *L. destructor*. By using a phage display *L. destructor* cDNA library [14], we could isolate two new IgE-binding clones showing high homology to tropomyosins and α -tubulins from many organisms. The *L. destructor* tropomyosin, which was named Lep d 10, was not selected during biopanning of the *L. destructor* cDNA library, which is described elsewhere [14]. A possible explanation is that the sera used for biopanning lacked a sufficient amount of allergen-specific IgE to detect Lep d 10 displayed on the phage in the library. However, in the present study, we could successfully PCR-amplify Lep d 10 from the *L. destructor* cDNA library. In immunoblot

analysis, both rLep d 10 and nLep d 10 were recognised by the same sera. Therefore, these data suggest that rLep d 10 is correctly folded in the bacterial expression system used in this study. Recombinant (r) Lep d 10 was shown to be a minor allergen with an IgE-binding frequency of 13%. This is in agreement with earlier data reporting rDer p 10 and rBlo t 10 as minor allergens with IgE-binding frequencies of 5.6% [20] and 20–29% [21], respectively. In contrast, Der f 10 was found to be a major allergen with an IgE-binding frequency of 80.6% among mite-sensitised individuals [19]. The large differences in the IgE-binding frequencies could be due to the geographically distinct populations screened in these studies being exposed to different repertoires of allergens.

Recently, five major IgE-binding regions were identified in the shrimp tropomyosin Pen a 1 [26]. These regions are homologous to regions of several other invertebrate tropomyosins, including Lep d 10. In agreement with IgE-binding regions from Pen a 1 [26], the Lep d 10 regions 187–202 and 247–284 contain, respectively, the amino acid sequences LEEEL and LEDEL, which are suggested to be allergenic motifs. Recombinant Lep d 10 was detected by the mAb 1A6, directed against *D. pteronyssinus* tropomyosin [25], which also recognises tropomyosins from shrimp [25], snail [27], and cockroach [28]. Furthermore, rLep d 10 was recognised by sera from 11 patients ImmunoCAP-positive to shrimp, but negative to *L. destructor*, indicating a cross-reactivity of Lep d 10 to other invertebrate tropomyosins. New sensitisations could also arise due to tropomyosin during immunotherapy with whole mite extracts. This has been observed during immunotherapy with *D. pteronyssinus* and *D. farinae* ex-

tracts, which induced or increased IgE reactivity against snail and shrimp [29–31]. In one of the studies, tropomyosin was identified as one of the cross-reactive allergens involved [29]. These findings stress the importance of diagnosing tropomyosin-specific IgE, and recombinant tropomyosin may be a useful tool in this respect.

The other finding of this study was the identification of a partial α -tubulin sequence as an IgE-binding protein from the *L. destructor* cDNA library. Sequence analysis of the full-length α -tubulin revealed the presence of one N-glycosylation site. Since no post-translational modifications are made in the *E. coli* expression system, the lack of sugar moieties might influence the protein folding, and hence the IgE reactivity, of the recombinant α -tubulin. In immunoblot experiments, we found that the recombinant α -tubulin, immobilised on the blotting membrane, was recognised by 12% of the individuals tested. Several of these *L. destructor* α -tubulin-positive sera were ImmunoCAP-negative to *L. destructor*, but ImmunoCAP-positive to *D. pteronyssinus* and/or crustaceans, implying that α -tubulin is a cross-reactive allergen. Our results from immunoblot analysis, clearly indicating α -tubulin as an IgE-binding protein, were not supported by the inhibition experiments. This could be due to different IgE-binding epitopes being exposed or masked when the protein is in solution compared to when it is immobilised on membrane. Interestingly, by using two-dimensional PAGE, α -

tubulin from *B. tropicalis* and *D. farinae* were recently identified as IgE-binding components [Dr. F.T. Chew, pers. commun.]. Characterisation, sequencing and expression of these α -tubulins are currently ongoing.

In conclusion, we have identified and cloned two new putative minor allergens, Lep d 10 (a tropomyosin) and α -tubulin, in the dust mite *L. destructor*. Both are highly conserved structural proteins that are present in all eukaryotic cells. α -Tubulin has so far not been reported to be an allergen in any other organism, while tropomyosin has been identified as an allergen in a large number of invertebrate species. Finally, the clinical relevance of these two allergens remains to be evaluated in vivo.

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