

Original article

Characterization of Der p 21, a new important allergen derived from the gut of house dust mites*

Background: The house dust mite (HDM) *Dermatophagoides pteronyssinus* is a major allergen source eliciting allergic asthma. The aim of the study was to identify new important HDM allergens associated with allergic asthma.

Methods: A cDNA coding for a new mite allergen, designated Der p 21, was isolated using immunoglobulin E (IgE) antibodies from patients with allergic asthma out of a *D. pteronyssinus* expression cDNA library and expressed in *Escherichia coli*.

Results: Circular dichroism analysis of the purified allergen showed that rDer p 21 (14 726 Da) is one of the few mite allergens with an α -helical secondary structure. The protein exhibited high thermal stability and refolding capacity, and, as determined by small angle X-ray scattering, formed a dimer consisting of two flat triangles. rDer p 21 bound high levels of patients' IgE antibodies and showed high allergenic activity in basophil activation experiments. Rabbit anti-Der p 21 IgG antibodies inhibited mite-allergic patients' IgE binding and allowed the ultrastructural localization of the allergen in the midgut (epithelium, lumen and faeces) of *D. pteronyssinus* by immunogold electron microscopy. Der p 21 revealed sequence homology with group 5 mite allergens, but IgE and IgG reactivity data and cross-inhibition studies identified it as a new mite allergen.

Conclusions: Der p 21 is a new important mite allergen which is liberated into the environment via faecal particles and hence may be associated with allergic asthma.

M. Weghofer¹, Y. Dall'Antonia²,
M. Grote³, A. Stöcklinger⁴,
M. Kneidinger⁵, N. Balic⁶, M.-T.
Krauth⁵, E. Fernández-Caldas⁷, W. R.
Thomas⁸, M. van Hage⁹, S. Vieths¹⁰,
S. Spitzauer⁶, F. Horak¹¹,
D. I. Svergun¹², P. V. Konarev¹²,
P. Valent⁵, J. Thalhammer⁴, W. Keller²,
R. Valenta¹, S. Vrtala¹

¹Division of Immunopathology, Department of Pathophysiology, Center for Physiology and Pathophysiology, Medical University of Vienna, Vienna, Austria; ²Division of Structural Biology, Institute of Chemistry, Karl-Franzens University, Graz, Austria; ³Institute of Medical Physics and Biophysics, University of Muenster, Muenster, Germany; ⁴Division of Allergy and Immunology, Department of Molecular Biology, University of Salzburg, Salzburg, Austria; ⁵Division of Hematology and Hemostaseology, Department of Internal Medicine I, Medical University of Vienna, Vienna, Austria; ⁶Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Vienna, Austria; ⁷Laboratorios LETI, S.L., Madrid, Spain; ⁸Center for Child Health Research, University of Western Australia, Telethon Institute of Child Health Research, West Perth, WA, Australia; ⁹Department of Medicine, Clinical Immunology and Allergy Unit, Karolinska Institutet, Stockholm, Sweden; ¹⁰Division of Allergology, Paul-Ehrlich-Institut, Langen, Germany; ¹¹Department of Otorhinolaryngology, Medical University of Vienna, Vienna, Austria; ¹²EMBL, Hamburg Outstation and Institute of Crystallography, Russian Academy of Sciences, Moscow, Russia

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Susanne Vrtala
Division of Immunopathology
Department of Pathophysiology
Center for Physiology and Pathophysiology
Medical University of Vienna
Waehringer Guertel 18-20, 1090 Vienna, Austria

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Abbreviations: cDNA, complementary deoxyribonucleic acid; HDM, house dust mite; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; MM, molecular mass; PFU, plaque forming units; RBL, rat basophil leukaemia; SAXS, small angle X-ray scattering; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; UV-CD, ultraviolet circular dichroism.

More than 40 years ago mites were discovered as major allergen sources in house dust (1). Today, mites are recognized as one of the most common causes of allergy worldwide, against which more than 50% of allergic patients are sensitized (2). Mite allergen exposure leads to the development of severe manifestations of allergy such as asthma (3). The house dust mites (HDMs) *Dermatophagoides pteronyssinus* and *farinae* frequently occur in the indoor environment in house dust and are considered as the most important sources of mite allergy (4). The allergens of *D. pteronyssinus* and *farinae* comprise more than 30 proteins or glycoproteins that show extensive immunological cross-reactivity (5). Since the isolation of a cDNA coding for the major HDM allergen, Der p 1, 20 different groups of mite allergens have been identified and characterized at the molecular level (6, 7). House dust mite allergens belong to different classes of proteins, among them enzymes and proteases as well as muscle or cytoskeletal proteins (7).

Approximately 30% of the HDM-allergic patients exhibit strong immunoglobulin E (IgE) reactivity to mite species other than HDMs such as storage mites (8). Studies performed with allergen extracts suggest that there are cross-reactive as well as species-specific allergens in *Dermatophagoides* species and nonpyroglyphid mites (*Lepidoglyphus destructor*, *Glycyphagus domesticus*, *Tyrophagus putrescentiae*) (9, 10). Therefore, mite allergic patients may be either cross-sensitized or co-sensitized to house dust and storage mites and hence diagnostic tools are required to discriminate between sensitization to house dust and other mites.

In this study we searched for new HDM allergens by screening of a cDNA library constructed from *D. pteronyssinus* RNA with serum IgE from HDM allergic patients. Among the isolated cDNA clones, one clone, designated Der p 21, contained the cDNA coding for a new 14.7 kDa allergen. The Der p 21 cDNA was expressed as native-like protein in *Escherichia coli*, purified to homogeneity and characterized by biochemical, biophysical and immunological methods. Our results suggest that Der p 21 is an important allergen which might be used for diagnosis and treatment of HDM allergy.

Materials and methods

Human sera, allergens and rabbit antiserum

Sera were obtained from mite allergic patients ($n = 200$) living in an urban area in Central Europe (Austria) with positive case history and perennial symptoms indicative for mite allergy, and mite-specific IgE antibodies (*D. pteronyssinus*-specific IgE > 0.7 kU_A/l) determined with the CAP-FEIA System (Phadia AB, Uppsala, Sweden).

Natural Der p 1 was isolated by affinity chromatography using the monoclonal antibody 4C1 (11). Der p 5 (GenBank accession number S76337) was expressed in *E. coli* and purified to homogeneity. Lep d 5 was expressed in *E. coli* and purified as described (12). Blot t 5 was a gift from Kaw Y. Chua, Department of Pediatrics, National University of Singapore.

A rabbit antiserum was raised against rDer p 21 using Freund's adjuvant (Charles River, Kisslegg, Germany).

Construction and IgE immunoscreening of a λ gt11 cDNA library

Dermatophagoides pteronyssinus were obtained from Allergon (Vällinge, Sweden). The cDNA library was constructed as described resulting in a λ gt11 expression cDNA library with 1.4×10^6 plaque forming units (PFU) (13). PFU (3×10^5) were immunoscreened with pooled serum IgE from mite-allergic patients and IgE-reactive clones were isolated as described (13). The serum pool consisted of five recently described sera (number I, V, VII, VIII, IX) with broad IgE reactivity to *D. pteronyssinus* allergens (14). The cDNA inserts of 40 IgE-reactive phage clones were polymerase chain reaction (PCR)-amplified using λ gt11 primers, sequenced (MWG, Ebersberg, Germany) and compared with sequences submitted to the GenBank database at the National Center for Biotechnology Information (NCBI).

Isolation of Der p 21 cDNA and sequence analysis

Phage DNA of one clone, designated Der p 21, was isolated using a liquid culture method (QIAGEN Lambda Midi Kit; Qiagen GmbH, Hilden Germany). The cDNA of Der p 21 was subcloned into the *KpnI/SacI* site of pUC 19, the ligation product transformed into *E. coli* XL1 Blue (13), and both DNA strands sequenced (MWG).

The deduced amino acid sequence was compared with the sequences deposited in the NCBI databases including SwissProt, PIR, PRF, PDB. Related allergens and their sequences were retrieved using the BLASTX program. Multiple sequence alignments were done with the program CLUSTALW 1.82 at the EBI server. The protein sequence of Der p 21 was analysed with tools of the ExPASy proteomics server and the protein secondary prediction was performed on the PSIPRED server.

Expression and purification of Der p 21 as recombinant allergen

The cDNA coding for the putative mature Der p 21 (nucleotide number 81-443) was PCR amplified using forward: 5'-GGAATTCCATATGTTTATTGTTGGTGA-3' and reverse: 5'-GAATTCCTAATAATATTCATCCGGAT-3' primers containing *EcoRI* and *NdeI* restriction sites (underlined), subcloned into the plasmid pET 17b (Novagen, EMD, Madison, WI, USA) and sequenced. Recombinant Der p 21 was expressed in *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA, USA) as described (15). After cell lysis (15), the soluble fraction, containing Der p 21, was dialysed against buffer A [10 mM Tris-Cl pH 7.0, 4% isopropanol, 1 ml/l phenylmethylsulphonylfluoride (PMSF)] and applied to a DEAE Sepharose Fast Flow column (GE Healthcare Bio-Sciences AB, Little Chalfont, UK). The recombinant allergen was eluted by a 0–500 mM NaCl gradient. Fractions containing the recombinant allergen were pooled, dialysed against buffer B (10 mM Na₂HPO₄ pH 4.0, 5 mM β -mercaptoethanol, 1 ml/l PMSF) and applied to a SP Sepharose Fast Flow column (GE Healthcare Bio-Sciences AB). The recombinant allergen was eluted by a combined pH 4.0–7.0 and 0–500 mM NaCl gradient and fractions containing more than 90% pure allergen were pooled and dialysed against 10 mM Na₂HPO₄, pH 7.0.

Matrix-assisted laser desorption ionization mass spectrometry and far-ultraviolet circular dichroism analysis of recombinant Der p 21

Purified Der p 21 was analysed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) (piChem,

Research and Development, Graz, Austria) as described (15).

The CD analysis of Der p 21 was performed in 15 mM potassium-phosphate buffer pH 6.9 at a concentration of 8.8×10^{-6} M on a Jasco J-715 spectropolarimeter (Tokyo, Japan) using a 0.1 cm path-length cell with cooling jacket connected to a water thermostating device. The sample was heated to 95°C with a heat rate of 1°C/min and cooled down at the same rate. A continuous temperature scan from 20°C to 95°C was performed at 222 nm with a step resolution of 0.5°C. Spectra in the wavelength range of 250 to 185 nm were recorded with 0.2 nm resolution at the scan speed of 50 nm/min and resulted from averaging three scans. The secondary structure content of the recombinant allergen was calculated using the program CDSSTR (16).

Small angle X-ray scattering experiments

The synchrotron radiation X-ray scattering data were collected on the X33 camera of the EMBL on the storage ring DORIS III (DESY, Hamburg, Germany). The data were recorded using a MAR345 two-dimensional image plate detector at a sample-detector distance of 2.7 m and a wavelength of $\lambda = 0.15$ nm, covering the range of momentum transfer $0.12 < s < 4.5 \text{ nm}^{-1}$ ($s = 4\pi \sin \theta / \lambda$, where 2θ is the scattering angle). To check for radiation damage, the data were collected in two successive 2-min exposures. The data were averaged after normalization to the intensity of the incident beam and the scattering of the buffer was subtracted. All data manipulations were performed using the program package PRIMUS. Der p 21 protein solution (in 10 mM HEPES buffer pH 7.0) was measured at two different concentrations (1.75 and 3.5 mg/ml), and no concentration-dependent change was observed. The low and high concentration data were merged for the subsequent data analysis. The forward scattering $I(0)$ and the radius of gyration (R_g) were evaluated using the Guinier approximation (17). These parameters, and the maximum diameter $D_{\max}$ of the particle were also computed from the entire scattering patterns using the program GNOM. The excluded volume V_p of the particle was computed from the Porod invariant (18). The molecular masses of the solutes were evaluated by comparison of the forward scattering with that from reference solutions of bovine serum albumin (MM = 66 kDa). The *ab initio* shape reconstruction was performed using the program GASBOR. The results from 10 separate runs were averaged to determine common structural features using the program DAMAVER. The structure of the Der p 21 dimer was further refined by rigid body modelling using the program SASREF in P2 symmetry, including contact information on the residues involved in the dimerization as restraints. The scattering amplitudes from the crystallographic model of α -spectrin (Protein Data Bank accession number 1CUN; spectrin repeat 16, residues 7–115) were calculated using the program CRY SOL (19). To enhance the model and compensate for missing amino acids, eight N- and six C-terminal residues were added using the program BUNCH. Two alternative approaches were used. The first approach assumed the monomer structure of α -spectrin to be fixed whereas in the second approach the N-terminal α -helix (residues 7–32) was attached to the rest of the molecule by a flexible linker (residues 33–39) and formed the intermolecular dimer interface. Both models were generated using P2 symmetry.

Immunogold electron microscopy

Rabbit IgG antibodies specific for Der p 21 were isolated by affinity chromatography (ImmunoPure IgG Purification Kit; Pierce, Rockford, IL, USA) from the antiserum of an immunized rabbit. The preimmune Igs were used as control. Mites were grown in 150 cm² vented cell culture flasks (Corning Incorporated, Corning, NY, USA)

under controlled humidity (75% relative humidity) and temperature (25°C) conditions. Adult *D. pteronyssinus* mites were pierced with a fine needle for penetration of fluids and fixed for 6 days at 4°C with 6% (v/v) *p*-formaldehyde, 0.5% (v/v) glutaraldehyde in 0.2 M PIPES buffer, pH 7.3. After dehydration in an ascending series of ethanol, the mites were embedded in Lowicryl K4M resin polymerized at –35°C using ultraviolet irradiation. Ultrathin sections (silver colour) were placed on nickel grids and were exposed successively to the following solutions [room temperature, moist chamber, phosphate-buffered saline (PBS) buffer/0.1% (w/v) bovine serum albumin (BSA), pH 7.3, Tris buffer/0.1% (w/v) BSA, pH 8.2]: (1) 5% (w/v) BSA in PBS buffer, 15 min; (2) rabbit anti-Der p 21 antibodies, diluted 1 : 100 in PBS buffer, 2 h; (3) PBS buffer, 2 $\times$ 5 min, Tris buffer, 1 $\times$ 5 min; (4) goat anti-rabbit IgG antibodies coupled to 10 nm colloidal gold particles (BritishBioCell; Plano, Wetzlar, Germany), 1 h; (5) Tris buffer, 1 $\times$ 5 min, distilled water, 2 $\times$ 5 min. Controls were run (1) by replacing the specific anti-Der p 21 antibodies by the preimmune antibodies and (2) by omitting the anti-Der p 21 antibodies from the labelling protocol. After immunolabelling, sections were stained with uranyl acetate and lead citrate and analysed in a Hitachi H 500 TEM (Nissei Sangyo, Tokyo, Japan) and Philips EM 410 (FEI Company, Eindhoven, The Netherlands) at 75 and 80 kV, respectively.

Measurement of rDer p 21 and nDer p 1-specific serum IgE levels by Streptavidin ImmunoCAP

rDer p 21 and nDer p 1 were dialysed against a carbonate buffer (0.1 M NaHCO₃, 1 M NaCl) and biotinylated for 3 h at room temperature with a fivefold molar excess of biotinamidohexanoyl-6-amino-hexanoic acid *N*-hydroxy-succinimide ester (NHS-Biotin; Sigma, St Louis, MO, USA) (20). Un-reacted biotin was removed by extensive dialysis against PBS. Prewashed Streptavidin ImmunoCAPs (Phadia AB) were loaded with 50 μ l biotinylated allergen (100 μ g/ml), incubated for 30 min, washed and used for the measurement of allergen-specific IgE by ImmunoCAP 100^e (Phadia AB). Serum IgE levels specific for rDer p 21 and nDer p 1 were determined in sera from 13 mite-allergic patients containing IgE antibodies both to rDer p 21 and nDer p 1 and, for control purposes, in serum from a nonallergic individual by ImmunoCAP measurements (Phadia AB).

IgE binding assays and IgE inhibition experiments

The frequency of IgE reactivity to Der p 21 was analysed in a nondenaturing dot blot assay using sera from mite-allergic patients and from one nonallergic individual as described (14).

Der p 21 and Der p 5 double-positive sera ($n = 9$) were used to study cross-reactivities between Der p 21 and Der p 5. Thirty Der p 21-positive sera were tested for IgE reactivity to rLep d 5 and rBlo t 5. Among the latter, six Lep d 5 and Der p 21-double-positive sera and three Blo t 5 and Der p 21 double-positive sera were found which could be used for inhibition experiments. Inhibitions were performed by pre-adsorbing 1 : 10 diluted sera with 10 μ g/ml protein at 4°C overnight. Nitrocellulose dotted proteins (0.5 μ g/dot) were incubated with the pre-adsorbed sera and bound IgE antibodies were detected as described (14).

Basophil activation assays (histamine release assay, rat basophil leukaemia cell mediator release and CD203c expression)

Peripheral blood mononuclear leucocytes from a *D. pteronyssinus*-allergic individual were incubated with nDer p 1, rDer p 5 or

rDer p 21 (10^{-6} – $1 \mu\text{g/ml}$) and histamine release was measured in triplicates as described (21).

Upregulation of CD203c expression on basophils after allergen exposure (rDer p 21, rDer p 5, rBlo t 5, rLep d 5) was measured as described (22).

For the β -hexosaminidase release assays, rat basophil leukaemia (RBL)-2H3 (RBL-703/21 transfected with the cDNA coding for the human high-affinity IgE receptor chain) cells were plated in 96-well tissue culture plates ($1 \times 10^5/\text{well}$) (23). After passive sensitization of the cells with mite-allergic patients' sera with IgE to Der p 21 and Der p 1 (dilution 1 : 30), cell degranulation was induced by adding 0.01–100 ng/ml nDer p 1 or rDer p 21. The β -hexosaminidase release was measured as described (24).

ELISA assay

rDer p 21, rDer p 5, rLep d 5 and rBlo t 5 ($5 \mu\text{g/ml}$ in PBS) were coated on an ELISA plate (Nunc, Roskilde, Denmark), incubated with 1 : 10 000 diluted rabbit anti-Der p 21 antiserum and the corresponding pre-immune serum and bound Ig antibodies were detected with 1 : 2000 diluted HRP-conjugated donkey anti-rabbit Ig antibodies (GE Healthcare Bio-Sciences AB) as described (15).

Mite extracts and immunoblots

Allergen extracts from *Glycyphagus domesticus*, *Lepidoglyphus destructor*, *Blomia tropicalis*, *Tyrophagus putrescentiae*, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* (Allergon) were prepared by homogenizing 0.3 g mites in 5 ml SDS buffer as described (25). Equal amounts of each extract were separated by 14% SDS-PAGE and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany). Membranes were incubated with rabbit antibodies specific for Der p 21 and the corresponding preimmune serum (dilution 1 : 1000) and bound antibodies were detected as described (26).

Results

Isolation and characterization of a cDNA coding for Der p 21

A. D. pteronyssinus λ gt11 cDNA library was immunoscreened with a serum pool from mite-allergic patients.

From the IgE-reactive clones, one clone which coded for a new mite allergen according to sequence analysis was selected for further characterization. The sequence analysis revealed that the cDNA contained a 420 base pair open reading frame encoding a 140 amino acid polypeptide with a calculated molecular mass of 16 531 Da (GenBank accession number DQ354124). The N-terminal sequence of 19 amino acids of Der p 21 corresponded to a signal peptide. According to computer-aided prediction and according to sequence similarity with mature Der p 5, the mature Der p 21 is assumed to start with the phenylalanine at position 20 (27). The mature protein including the start codon contained 122 amino acid residues with a calculated MM of 14 726 Da and an isoelectric point of 5.25. Amino acid similarity searches revealed that the deduced amino acid sequence of Der p 21 showed similarity to group 5 allergens of other mite species (Fig. 1A). Closer sequence identity (49%) was observed with the group 5 allergen from *Lepidoglyphus destructor* (NCB accession number Q9U5P2). Significant identity was also noted with the group 5 allergens from *Blomia tropicalis* (NCB accession number O96870) (41%) and *Dermatophagoides pteronyssinus* (NCB accession number P14004) (32%). No significant homologies with other known proteins and domains were found.

Expression and purification of the recombinant allergen

Der p 21 was expressed as nonfusion protein in the soluble fraction of *E. coli* extracts. Using several consecutive purification steps, a protein migrating at approximately 14–15 kDa was obtained (Fig. 1B). The MM of the purified protein determined by MALDI-MS (14 725 Da) (Fig. 1C) is in agreement with the protein migration in SDS-PAGE and the MM calculated from the deduced aa sequence (14 726 Da).

Analysis of the far-ultraviolet circular dichroism (UV-CD) spectrum of Der p 21 at room temperature showed a

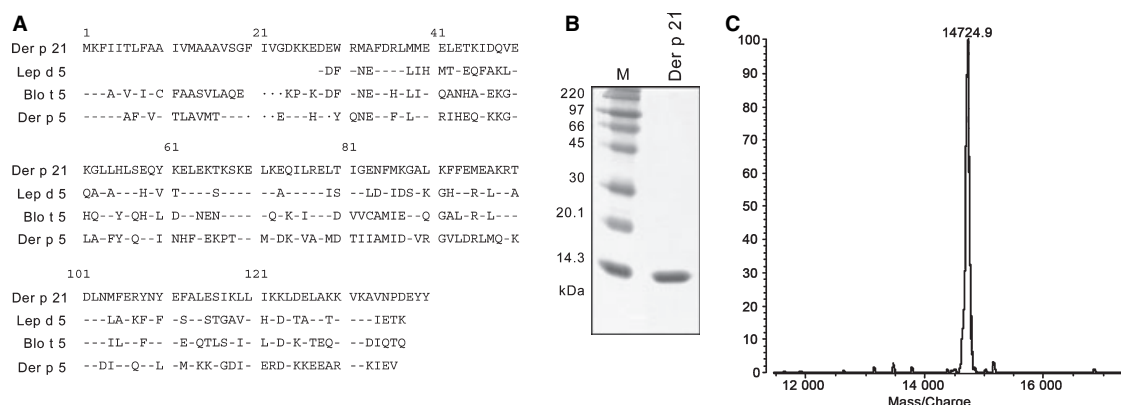


Figure 1. Purified rDer p 21. (A) Der p 21 was compared with group 5 allergens. Amino acids identical to Der p 21 are indicated with hyphens and points represent gaps. (B) SDS-PAGE gel containing 3 μg of purified rDer p 21 (M = molecular mass marker). (C) Mass spectroscopical analysis. The signal intensity is displayed on the y-axis.

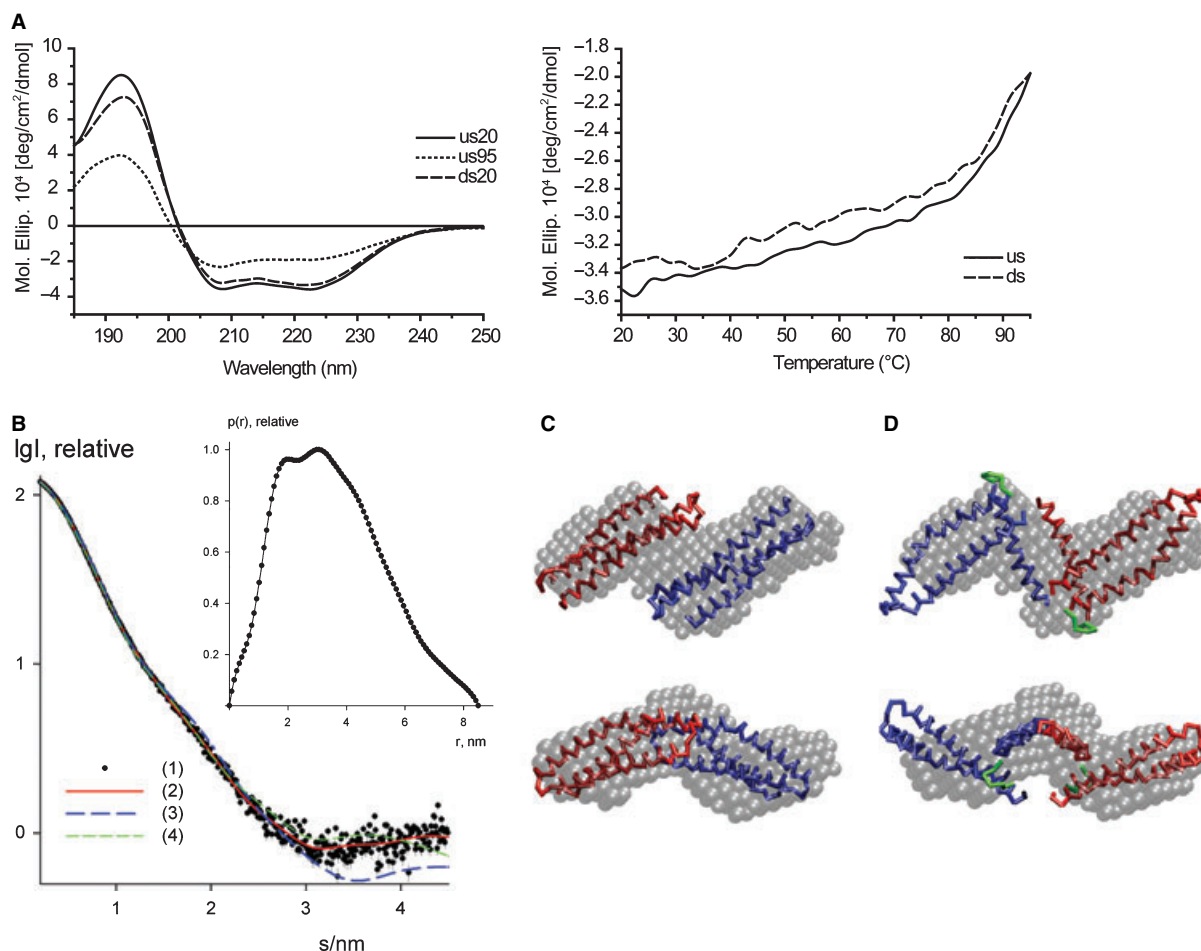


Figure 2. (A, left) UV-CD spectra at us20 = 20°C, us95 = 95°C and after cooling at ds20 = 20°C. (A, right) Thermal unfolding (*us*) and refolding curves (*ds*). (B) X-ray scattering pattern from Der p 21 (dots), from *ab initio* (full line), from the crystallographic triple-coiled (long dashed line) and from double coiled model (short dashed line). (C, D) Two *ab initio* bead models of Der p 21.

typical α -helical spectrum with two minima at 208 and 222 nm and a large maximum at 192.5 nm (Fig. 2A). Secondary structure analysis using the program CDSSTR with the reference data set 3 yielded 88% α -helix, 5% β -sheet, 2% turns and 4% random coil structure with an excellent fit (normalized root-mean-square deviation = 0.007). Upon heating the protein unfolded only partially even at 95°C (Fig. 2A, left). The transition temperature T_M could not be determined accurately due to the limited heating range, but was estimated to lie between 94 and 100°C according to the shape of the unfolding curve. Upon cooling to room temperature Der p 21 almost completely restored its structure, indicating high heat stability and refolding capacity (Fig. 2A).

Solution structure of Der p 21

In order to investigate the solution structure of Der p 21 we performed small angle X-ray scattering experiments (SAXS). The experimental SAXS curve from Der p 21 is displayed in Fig. 2B. The estimated MM of the protein,

26 ± 4 kDa, is compatible with a dimer structure. This finding is further corroborated by the excluded volume of the sample (50 ± 5 nm³), noting that for sufficiently large globular proteins the hydrated volume in nm³ should numerically be about twice the MM in kDa. The values of R_g (2.65 ± 0.15) nm and D_{max} (8.5 ± 0.5) nm exceed significantly those from the monomer part and also point to the dimer formation. According to the $p(r)$ function the shape of the dimer is rather elongated (Fig. 2B, inset). To reconstruct the shape of Der p 21 at low resolution, 10 *ab initio* models (discrepancies $\chi = 1.97 \div 2.19$ from the experimental curve) were calculated using the program GASBOR. The most typical *ab initio* model of Der p 21 according to the program DAMAVER is presented as an envelope in Fig. 2C,D. It is in agreement with the proposed elongated shape of the particle. The envelope of the averaged models adopts the shape of two flat triangles related by twofold symmetry. The maximum dimension of the envelope amounts to 8.7 nm. To further refine the structure of the Der p 21 dimer, rigid body modelling was performed in terms of two high-resolution

triple coiled-coil domain models related by twofold symmetry. The model consists of the spectrin repeat 16 of chicken α -spectrin (amino acids 7–115 of the crystal structure 1CUN). The missing eight N- and six C-terminal residues were added using the program BUNCH and the best model yielded a fit of comparable quality ($\chi = 2.39$) to the *ab initio* models (Fig. 2B). This model, obtained without distance constraints between monomers, overlaps well with the most typical *ab initio* shape, but has a quite small dimer interface area (Fig. 2C). In order to allow for a more intimate dimer interface, an alternative approach was applied in which the N-terminal helix (residues 7–32) was connected to the residual molecule by a flexible linker (residues 33–39) and was allowed to move during rigid-body refinement (Fig. 2D). This model yields an even better fit to the experimental data with $\chi = 1.75$ (Fig. 2B). In the refined dimer the N-terminal helices form an intermolecular anti-parallel dimeric coiled-coil, whereas helices 2 and 3 form intramolecular dimeric coiled-coils. In Fig. 2C,D the two models were superimposed onto the envelope generated from the averaged *ab initio* models.

Localization of Der p 21 in gut epithelial cells of HDMs by immunogold electron microscopy

Figure 3A shows a part of the whole body of *D. pteronyssinus* at low magnification. This part includes a cross-section of the midgut. The inner surface wall of the midgut is lined with epithelial cells (GE), which have numerous short microvilli (MI) facing the lumen. The lumen of the midgut is partly filled with food materials under digestion (DF). In Fig. 3B, a detail of an epithelial cell is shown at higher magnification. An extensive rough endoplasmic reticulum (ER) is seen stacked throughout the cell. A number of electron-dense droplet-like bodies (SD), probably transport vesicles, are observed in the cytoplasm of the cell. They contain numerous gold particles representing the sites of Der p 21 in the cell. Gold particles were also found associated with the cytoplasmic matrix, the endoplasmic reticulum and the microvilli (MI). Figure 3C shows a detail of the microvilli adjacent to the gut lumen at high magnification. Large clusters of gold particles were associated with microvilli and irregularly shaped cytoplasmic material that was found in the lumen of the gut between the microvilli and the food under digestion. Few gold particles were found in the digested food. Only very few nonspecifically adsorbed gold particles were found after incubation with the control antibodies (Fig. 3D).

Der p 21 is an important, potent and new HDM allergen distinct from group 5 mite allergens

rDer p 21 was tested for its IgE binding capacity by dot-blot analysis in a first population of 117 *D. pteronyssinus* allergic patients. Der p 21-specific IgE antibodies were

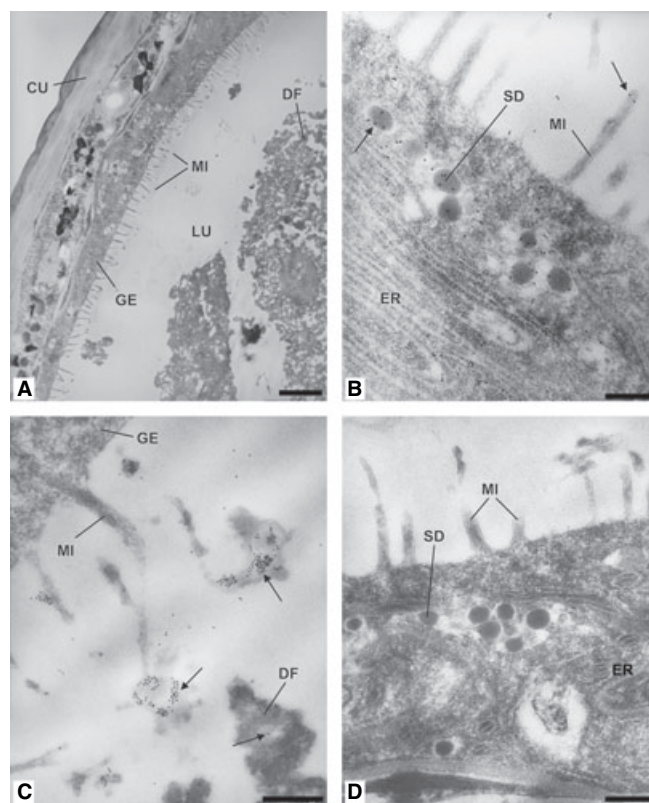


Figure 3. Localization of Der p 21. (A) Gut, (B, C) details of the gut epithelium and lumen after incubation with anti-Der p 21 antibodies and (D) with control antibodies. Arrows point to gold particles (black dots). GE, gut epithelium; MI, microvilli; LU, lumen; DF, digested food; CU, cuticle layer; ER, rough endoplasmic reticulum; SD, electron-dense bodies. Bars: A, 1 μ m; B–D, 0.3 μ m.

detected in 30 *D. pteronyssinus*-allergic patients' sera (26%). In comparison, 31% of the patients exhibited serum IgE reactivity to rDer p 5 (36 patients) and 21% recognized both, rDer p 21 and rDer p 5 (24 patients). Five per cent of the patients showed IgE reactivity to rDer p 21 but not to rDer p 5 (six patients) and 10% reacted with rDer p 5 and not with rDer p 21 (12 patients).

The amount of IgE antibodies directed to Der p 21 and Der p 1 was determined by allergen-specific Streptavidin ImmunoCAP. Thirteen mite-allergic patients with IgE reactivity to Der p 21 and Der p 1 were tested with Der p 21- and Der p 1-specific ImmunoCAPs (Table 1). IgE antibody levels to Der p 21 were between 2.57 and 95.9 kU_A/l (Class 2–5) and levels to Der p 1 were between 5.33 and >100 kU_A/l (Class 3–6). Serum from a nonallergic individual showed no IgE reactivity.

As Der p 21 showed sequence homology to the group 5 allergens from different dust mites, we investigated whether there is IgE cross-reactivity between Der p 21 and group 5 allergens (Der p 5, Lep d 5, Blo t 5). As mentioned before, from the 30 Der p 21-positive patients, six did not react with Der p 5 and from the 36

Table 1. IgE antibody levels (kU_A/l) specific for rDer p 21 and nDer p 1

Patient	rDer p 21		nDer p 1	
	Conc (kU _A /l)	Class	Conc (kU _A /l)	Class
1	14.60	3	11.70	3
2	65.40	5	144.00	6
3	54.40	5	144.00	6
4	47.50	4	30.30	4
5	8.61	3	10.30	3
6	72.80	5	136.00	6
7	2.86	2	15.50	3
8	8.46	3	11.20	3
9	34.70	4	80.50	5
10	2.57	2	5.33	3
11	95.90	5	147.00	6
12	36.50	4	88.30	5
13	9.29	3	36.30	4
Mean	34.89		66.19	
NC	<0.35	0	<0.35	0

kU_A/l, kilo units antigen/litre. NC, negative control (nonallergic individual).

Der p 5-positive patients 12 did not react with Der p 21. In order to study cross-reactivity between Der p 21 and Der p 5, patients' sera containing IgE antibodies to Der p 21 and Der p 5 were pre-incubated with rDer p 21 or rDer p 5, which caused complete auto-inhibition of IgE binding to the allergen (Fig. 4A). rDer p 5 did not cause relevant inhibition of IgE binding to Der p 21 and vice versa in eight of nine sera demonstrating that Der p 21 and Der p 5 are independent allergens.

Cross-reactivity between Der p 21, Lep d 5 and Blo t 5 was studied with additional 30 Der p 21-reactive sera. Only six sera reacted with Lep d 5 and even less ($n = 3$) showed IgE reactivity to Blo t 5. With the exception of one serum (patient 5), reactivity of these sera to Lep d 5 and Blo t 5 was much weaker than to Der p 21 (Fig. 4B,C). A relevant inhibition of IgE reactivity to Lep d 5 and Blo t 5 by Der p 21 was only observed in one patient for Blo t 5 (patient 6) and in one patient for Lep d 5 (patient 21) (Fig. 4B,C) whereas neither Lep d 5 nor Blo t 5 induced a relevant inhibition of IgE reactivity to Der p 21.

rDer p 21 shows allergenic activity in basophil activation experiments

The allergenic activity of rDer p 21 was evaluated by basophil degranulation experiments (Fig. 5). As shown for a mite-allergic patient containing Der p 21-, Der p 5- and Der p 1-reactive IgE antibodies, Der p 21 and Der p 5 induced maximal histamine release already at very low allergen concentrations (10^{-5} – 10^{-4} µg/ml) (Fig. 5A, left), whereas nDer p 1 induced maximal histamine release at 10^{-3} µg/ml. Similar results were obtained when the allergenic activity of Der p 21 was assessed with RBL cells that had been transfected with the human FcεRI (Fig. 5B). Cells were loaded with mite-allergic patients'

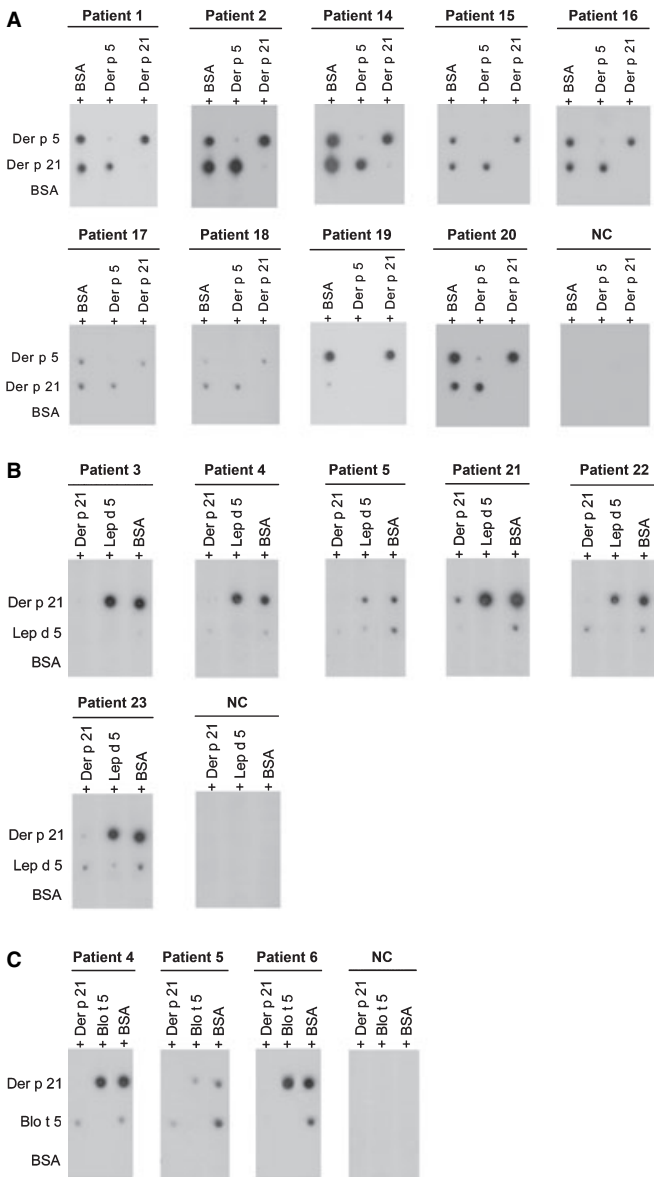


Figure 4. Lack of relevant IgE cross-reactivity between Der p 21 and group 5 allergens. Patients with simultaneous IgE reactivity to (A) Der p 21 and Der p 5, (B) Der p 21 and Lep d 5, (C) Der p 21 and Blo t 5 were pre-adsorbed with proteins (top) and then exposed to nitrocellulose-dotted proteins (left). NC, non-allergic individual.

IgE and stimulated with various concentrations of Der p 21 and Der p 1. nDer p 1 was more allergenic than rDer p 21 in two patients, but rDer p 21 exhibited allergenic activity which was comparable (patient 21) or even better (patient 25) than that of nDer p 1 in certain patients (Fig. 5B).

Basophils from additional mite-allergic patients (patients 2, 7, 8) were incubated with various concentrations of Der p 21, Der p 5, Lep d 5 and Blo t 5 and the upregulation of the basophil activation marker CD203c was determined (Fig. 5A). Der p 21 as well as Der p 5

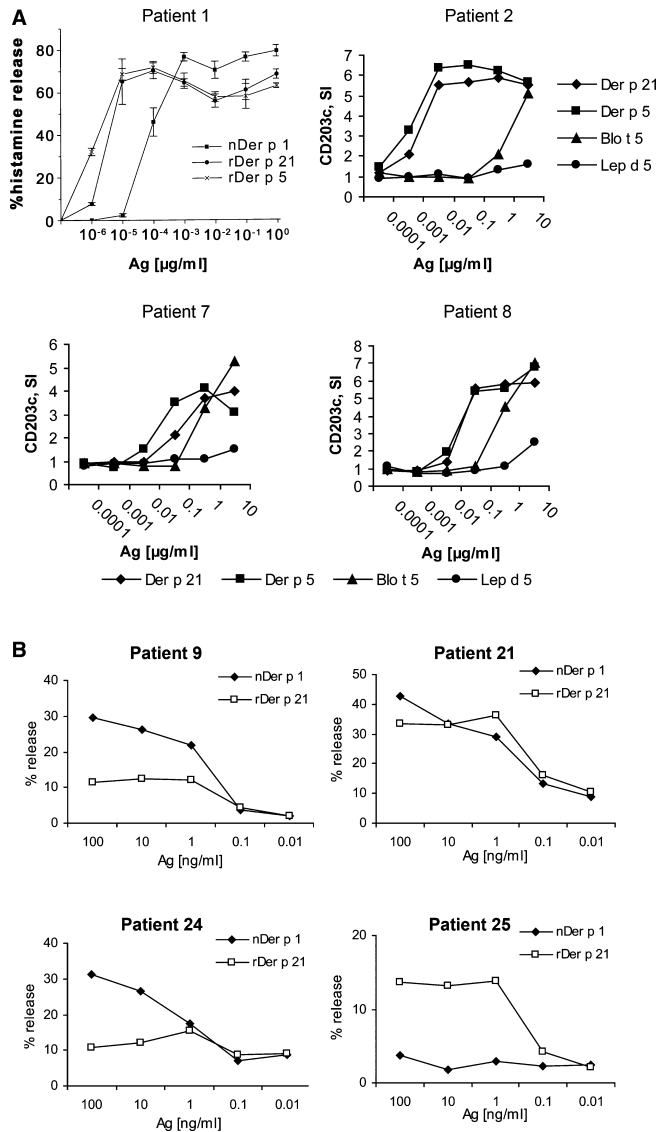


Figure 5. Allergenic activity of rDer p 21. (A) Induction of specific histamine release (patient 1), upregulation of CD203c expression (patients 2, 7, 8) and (B) induction of β-hexosaminidase release from humanized RBL cells sensitized with sera from patients 9, 21, 24 and 25. SI, stimulation index; Ag, allergen.

induced upregulation of CD203c expression at concentrations between 1 and 100 ng/ml. Lep d 5 did not induce upregulation of CD203c expression up to a concentration of 1 μg/ml, whereas incubation of basophils with 1 μg/ml of Blo t 5 induced CD203c upregulation in each of the patients.

Der p 21 represents a new allergen which is specific for *D. pteronyssinus* and *D. farinae*

Rabbit IgG antibodies raised by immunization with Der p 21 reacted strongly with Der p 21, but not with Der p 5

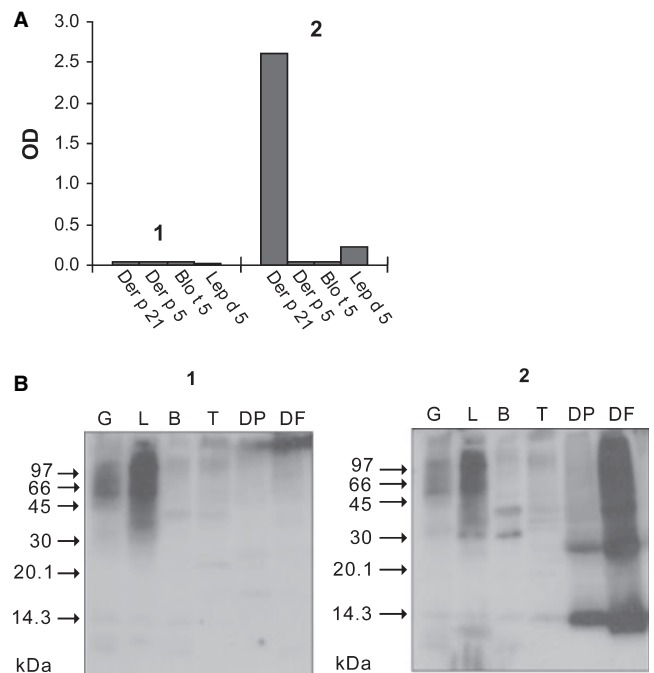


Figure 6. Anti-Der p 21 antibodies specifically detect the allergen in house dust mites. (A) Reactivity of Der p 21-specific IgG to mite allergens. (B) IgG-reactivity of Der p 21 specific antibodies with mite extracts. G, *Glycyphagus domesticus*; L, *Lepidoglyphus destructor*; B, *Blomia tropicalis*; T, *Tyrophagus putrescentiae*; DP, *Dermatophagoides pteronyssinus*; DF, *Dermatophagoides farinae*. Pre-immune- (1) and immune serum (2).

or Blo t 5, and only a very weak reactivity with Lep d 5 was observed. No reactivity was obtained with the pre-immune serum (Fig. 6A). To test whether homologous and cross-reactive allergens to Der p 21 are present in the mites *Glycyphagus domesticus*, *Lepidoglyphus destructor*, *Blomia tropicalis*, *Tyrophagus putrescentiae*, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, extracts of these mite species were tested with rabbit anti-Der p 21 serum (Fig. 6B). The rabbit serum reacted specifically at approximately 14 kDa with the HDMs *D. pteronyssinus* and *D. farinae* but showed almost no reactivity with storage mites and the tropical mite *B. tropicalis* in the 14 kDa region and with high molecular weight moieties > 28 kDa representing dimers and oligomers. This result indicates that Der p 21 is specific for HDMs.

Discussion

In the present study we have isolated a cDNA coding for a 14.7 kDa HDM allergen from a *Dermatophagoides pteronyssinus* expression cDNA library using serum IgE from *D. pteronyssinus*-allergic patients. Sequence analysis revealed that Der p 21 exhibits an average amino acid sequence identity of approximately 40% with group 5

mite allergens but it represents an independent HDM allergen, because no relevant IgE cross-reactivity between Der p 21 and Der p 5 could be detected. Among 30 Der p 21-positive sera only 6 and 3 showed weak IgE reactivity with Lep d 5 and Blo t 5, respectively and again no relevant cross-reactivity was detected. This finding suggests that Der p 21 is also an allergen distinct from Lep d 5 and Blo t 5.

The clinical importance of Der p 21 is underlined by two findings. First, we found in a European population of HDM-allergic patients that approximately the same percentage of patients showed IgE reactivity with Der p 21 as with Der p 5, an important mite allergen (28). Second, Der p 21 binds high levels of IgE antibodies from mite-allergic patients and had high allergenic activity.

Mite faeces have been identified as a major source for HDM allergens (2). Using immunogold electron microscopy we could localize Der p 21 in the microvillous midgut of HDMs. We found that Der p 21 is associated with the endoplasmatic reticulum, electron-dense droplets which represent presumably transport vesicles, the cytoplasmic matrix, microvilli and also with detached cytoplasmatic material. Furthermore, we found that Der p 21 was also associated with digested food indicating that the allergen is mainly liberated via mite faeces.

The CD analysis of Der p 21 revealed that the allergen represents a heat-stable protein and one of the few HDM allergens known today with an exclusive α -helical secondary structure. Stability is a common feature for several allergens and is a crucial factor for allergenicity of proteins because it allows these proteins to survive in the environment in a folded manner. Coiled-coil regions were predicted for the entire α -helical sequence range. Based on these predictions it was suggested that Der p 5 and other group 5 allergens adopt a triple coiled-coil structure (19, 29). Due to the high sequence identity with group 5 mite allergens and the conserved heptad-repeats we assumed that Der p 21 also forms a triple coiled-coil structure.

The triple-coiled model as well as a double-coiled model fit the envelope reasonably well. However, given that the former model displays very poor contacts between the triple-coiled monomers, the double-coiled model appears to be more probable. It is interesting in this context, that many coiled-coil proteins have been identified as strong antigens and that roughly a third of

known human auto-antigens were predicted to contain coiled-coil regions (30).

We found that only few Der p 21-positive sera showed weak IgE reactivity to Blo t 5 and Lep d 5 and there was no relevant cross-reactivity in the few double-positive sera indicating that reactivity to Der p 21 can discriminate HDM-allergic patients from patients sensitized to storage mites or other mite species. This assumption is also supported by the finding that even a high titre polyclonal rabbit antiserum raised against rDer p 21 showed no relevant cross-reactivity with Lep d 5 and Blo t 5 or with extracts from storage mites or tropical mites.

Whereas Lep d 5 showed no significant upregulation of the activation marker CD203c, Blo t 5 induced activation of basophils from Der p 21-allergic patients at high concentrations. This low reactivity to Blo t 5 could be due to cross-reactivity, but it is more likely caused by co-sensitization to *Blomia* spp. Sensitization to *Blomia* spp. has been described in many countries including in German city dwellers and is not only limited to tropical and subtropical environments (31). Different species of *Blomia*, including *B. tropicalis*, have been found in dust samples in countries with a temperate climate (e.g. Germany) (32).

The first reports of successful immunotherapy treatment of allergic patients with recombinant allergens or recombinant allergen derivatives have been published recently (33, 34). For complex allergen sources such as grass pollen, therapeutic vaccines have been applied which consist of mixtures of the most important allergens.

Based on our results, it may also be considered to incorporate Der p 21 in a therapeutic vaccine against HDM allergy.

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References

1. Voorhorst R, Spieksma FT, Varekamp H, Leupen MJ, Lyklema AW. The house dust mite (*Dermatophagoides pteronyssinus*) and the allergens it produces. Identity with the house-dust allergen. *J Allergy* 1967;**39**:325–339.
2. Tovey ER, Chapman MD, Platts-Mills TA. Mite faeces are a major source of house dust allergens. *Nature* 1981;**289**:592–593.
3. Platts-Mills TA, Rakes G, Heymann PW. The relevance of allergen exposure to the development of asthma in childhood. *J Allergy Clin Immunol* 2000;**105**:S503–S508.
4. Fernandez-Caldas E. Mite species of allergologic importance in Europe. *Allergy* 1997;**52**:383–387.
5. Baldo BA, Ford SA, Tovey ER. Toward a definition of the complete spectrum and rank order of importance of the allergens from the house dust mite: *Dermatophagoides pteronyssinus*. *Adv Biosci* 1989;**74**:13–31.

6. Chua KY, Stewart GA, Thomas WR, Simpson RJ, Dilworth RJ, Plozza TM et al. Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. *J Exp Med* 1988;**167**:175–182.
7. Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brien RM. Characterization and immunobiology of house dust mite allergens. *Int Arch Allergy Immunol* 2002;**129**:1–18.
8. Olsson S, van Hage-Hamsten M. Allergens from house dust and storage mites: similarities and differences, with emphasis on the storage mite *Lepidoglyphus destructor*. *Clin Exp Allergy* 2000;**30**:912–919.
9. van der Heide S, Niemeijer NR, Hovenga H, de Monchy JG, Dubois AE, Kauffman HF. Prevalence of sensitization to the storage mites *Acarus siro*, *Tyrophagus putrescentiae*, and *Lepidoglyphus destructor* in allergic patients with different degrees of sensitization to the house-dust mite *Dermatophagoides pteronyssinus*. *Allergy* 1998;**53**:426–430.
10. Johansson E, Borga A, Johansson SG, Van Hage-Hamsten M. Immunoblot multi-allergen inhibition studies of allergenic cross-reactivity of the dust mites *Lepidoglyphus destructor* and *Dermatophagoides pteronyssinus*. *Clin Exp Allergy* 1991;**21**:511–518.
11. Hales B J, Shen HD, Thomas WR. Cytokine responses to Der p 1 and Der p 7: house dust mite allergens with different IgE-binding activities. *Clin Exp Allergy* 2000;**30**:934–943.
12. Eriksson TL, Rasool O, Huecas S, Whitley P, Crameri R, Appenzeller U et al. Cloning of three new allergens from the dust mite *Lepidoglyphus destructor* using phage surface display technology. *Eur J Biochem* 2001;**268**:287–294.
13. Vrtala S, Sperr WR, Reimitzer I, van Ree R, Laffer S, Muller WD et al. cDNA cloning of a major allergen from timothy grass (*Phleum pratense*) pollen; characterization of the recombinant Phl pV allergen. *J Immunol* 1993;**151**:4773–4781.
14. Weghofer M, Thomas WR, Pittner G, Horak F, Valenta R, Vrtala S. Comparison of purified *Dermatophagoides pteronyssinus* allergens and extract by two-dimensional immunoblotting and quantitative immunoglobulin E inhibitions. *Clin Exp Allergy* 2005;**35**:1384–1391.
15. Swoboda I, Bugajska-Schretter A, Verdino P, Keller W, Sperr WR, Valent P et al. Recombinant carp parvalbumin, the major cross-reactive fish allergen: a tool for diagnosis and therapy of fish allergy. *J Immunol* 2002;**168**:4576–4584.
16. Whitmore L, Wallace BA. DICHRO-WEb, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Res* 2004;**32**:W668–W673.
17. Guinier A. La diffraction des rayons X aux tres petit angles: Application à l'étude de phénomènes ultramicroscopique. *Ann Phys (Paris)* 1939;**12**:161–237.
18. Porod G. General theory. In: Glatter O, Kratky O, editors. Small-angle X-ray scattering. London: Academic Press, 1982:17–51.
19. Grum VL, MacDonald RI, Mondragon A. Structures of two repeats of spectrin suggest models of flexibility. *Cell* 1999;**98**:523–535.
20. Erwin EA, Custis NJ, Satinover SM, Perzanowski MS, Woodfolk JA, Crane J et al. Quantitative measurement of IgE antibodies to purified allergens using streptavidin linked to a high-capacity solid phase. *J Allergy Clin Immunol* 2005;**115**:1029–1035.
21. Valent P, Besemer J, Muhm M, Majdic O, Lechner K, Bettelheim P. Interleukin 3 activates human blood basophils via high-affinity binding sites. *Proc Natl Acad Sci U S A* 1989;**86**:5542–5546.
22. Hauswirth AW, Natter S, Ghannadan M, Majlesi Y, Scherthaner GH, Sperr WR et al. Recombinant allergens promote expression of CD203c on basophils in sensitized individuals. *J Allergy Clin Immunol* 2002;**110**:102–109.
23. Vogel L, Luttkopf D, Hatahet L, Hausteiner D, Vieths S. Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* 2005;**60**:1021–1028.
24. Hartl A, Hochreiter R, Stepanoska T, Ferreira F, Thalhammer J. Characterization of the protective and therapeutic efficiency of a DNA vaccine encoding the major birch pollen allergen Bet v 1a. *Allergy* 2004;**59**:65–73.
25. Vrtala S, Grote M, Duchêne M, van Ree R, Kraft D, Scheiner O et al. Properties of tree and grass pollen allergens: reinvestigation of the linkage between solubility and allergenicity. *Int Arch Allergy Immunol* 1993;**102**:160–169.
26. Valenta R, Duchêne M, Ebner C, Valent P, Sillaber C, Deviller P et al. Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992;**175**:377–385.
27. Zhang Z, Henzel WJ. Signal peptide prediction based on analysis of experimentally verified cleavage sites. *Protein Sci* 2004;**13**:2819–2824.
28. Lynch NR, Thomas WR, Garcia NM, Di Prisco MC, Puccio FA, L'Opez RI et al. Biological activity of recombinant Der p 2, Der p 5 and Der p 7 allergens of the house-dust mite *Dermatophagoides pteronyssinus*. *Int Arch Allergy Immunol* 1997;**114**:59–67.
29. Liaw SH, Chen HZ, Liu GG, Chua KY. Acid-induced polymerization of the group 5 mite allergen from *Dermatophagoides pteronyssinus*. *Biochem Biophys Res Commun* 2001;**285**:308–312.
30. Dohlman JG, Lupas A, Carson M. Long charge-rich alpha-helices in systemic autoantigens. *Biochem Biophys Res Commun* 1993;**195**:686–696.
31. Fernández-Caldas E, Lockey RF. *Blomia tropicalis*, a mite whose time has come. *Allergy* 2004;**59**:1161–1164.
32. Franz JT, Masuch G, Müskén H, Bergmann KC. Mite fauna of German farms. *Allergy* 1997;**52**:1233–1237.
33. Jutel M, Jaeger L, Suck R, Meyer H, Fiebig H, Cromwell O. Allergen-specific immunotherapy with recombinant grass pollen allergens. *J Allergy Clin Immunol* 2005;**116**:608–613.
34. Niederberger V, Horak F, Vrtala S, Spitzauer S, Krauth MT, Valent P et al. Vaccination with genetically engineered allergens prevents progression of allergic disease. *Proc Natl Acad Sci USA* 2004;**101**(Suppl 2):14677–14682.