

Original article

Molecular cloning and characterization of full-length cDNAs encoding a novel high-molecular-weight *Dermatophagoides pteronyssinus* mite allergen, Der p 11

Background: *Dermatophagoides pteronyssinus* (Dp) and *D. farinae* (Df) mites are the most important source of indoor aeroallergens. Most Dp mite allergens identified to date have relatively low molecular weights (MWs). Identification of high-MW mite allergens is a crucial step in characterizing the complete spectrum of mite allergens and to provide appropriate tools for diagnostic and therapeutic application.

Methods: The full-length Der p 11 cDNA clone was isolated using cDNA library immunoscreening, the 5′–3′ rapid amplification of cDNA ends (RACE) system and polymerase chain reactions (PCR). The whole cDNA insert and its PCR-derived DNA fragments (p1 to p4) were generated and expressed in the *Escherichia coli* expression system. The allergenicity of the recombinant protein and its peptide fragments was examined by IgE immunodot assays. The IgE-binding reactivity of rDer p 11 was analyzed in the serum of 50 asthmatic children with positive reactivity to Dp mite extract. Its recombinant peptide fragments were also examined by immunodot assays in 30 mite-allergic children.

Results: Der p 11 cDNA consists of a 2625-bp open reading frame encoding a 103-kDa protein with 875 amino acids. It exhibits significant homology with the paramyosin of other invertebrates. The protein sequence alignment of this newly identified Dp mite allergen (denominated as Der p 11) revealed over 89% identity with Der f 11 and Blo t 1. Among 50 Dp-sensitive asthmatic children, rDer p 11 showed positive IgE-binding reactivity to 39 patients (78%). Using immunodot assays, multiple human IgE-binding activities were demonstrated in all four fragments of Der p 11. Using immunoblot assays, the dominant IgG-binding epitope for monoclonal antibody (mAb642) was located in fragment p3 only. In immunoblot assays, cross-inhibition between rDer p 11 and rDer f 11 was up to 73–80% at concentrations of 100 µg/ml.

Conclusions: This study confirms that the newly identified recombinant Der p 11 is a novel and important high-MW Dp mite allergen for asthmatic children. Our data also indicates that human IgE-binding major epitopes are scattered over the entire molecule of Der p 11.

L. C. Tsai¹, H. J. Peng¹, C. S. Lee²,
P. L. Chao¹, R. B. Tang³, J. J. Tsai⁴,
H. D. Shen¹, M. W. Hung¹, S. H. Han¹

¹Department of Medical Research and Education, Taipei-Veterans General Hospital, Taipei, Taiwan;

²Department of Internal Medicine, Mackay Memorial Hospital, Taipei, Taiwan; ³Department of Pediatrics, Taipei-Veterans General Hospital, Taipei, Taiwan; ⁴Section of Allergy and Clinical Immunology, Cathy General Hospital, Taipei, Taiwan

Key words: amino acid sequence; *Dermatophagoides pteronyssinus*; mite allergen; molecular cloning.

Lai-Chen Tsai
Department of Medical Research and Education
Taipei-Veterans General Hospital
Taipei
Taiwan

Accepted for publication 19 April 2004

Aeroallergens derived from house dust mites *Dermatophagoides pteronyssinus* (Dp) and *D. farinae* (Df) have been recognized as major triggering factors for bronchial asthma, perennial rhinitis and atopic dermatitis (1). Epidemiological studies revealed that mite allergy affects about 10–20% of world population (1). Using immunoblotting, it has been shown that there are more than 30 different IgE-binding components of mite proteins with molecular weights (MWs) ranging from 11 to over 100 kDa (2). Among 19 denominated mite allergens, the major human IgE-binding reactivity has been reported for the group 1, 2, 3, 9, 11, 14 and 15 allergens (3). Most mite allergens are proteins with MWs

lower than 60-kDa (3, 4). The cDNA clones coding for most low-MW allergens have been isolated (3, 5).

Previous studies have repeatedly demonstrated that several high-MW components of mite extracts possess significant IgE-binding activities to the sera of mite-allergic asthmatic patients (2, 6, 7). However, the studies of high-MW mite allergens have been relatively overlooked. Until recently, three groups of high-MW mite allergens Der p 11, 14 (both allergens for humans) and Der f 15 (allergen for dogs and cats) have been newly identified as major allergens (8–11). We have first reported that the paramyosin of *D. farinae*, Der f 11, has strong IgE-binding activities in many asthmatic

patients (8, 9). Moreover, *in vivo* skin tests and *in vitro* IgE-binding immunodot assays have shown 62% and 50% positive reactivity, respectively, to the recombinant 98-kDa polypeptides of Der f 11 (8, 9). Subsequently, the paramyosin of *Blomia tropicalis*, Blo t 11, has been also characterized to have multiple human IgE- and IgG-binding epitopes scattered throughout the molecule (12).

In this report, we defined the full-length cDNAs of a novel high-MW mite allergen from Dp by molecular cloning and sequence analysis. This Dp cDNA clone encodes a large protein with 875 amino acid residues. The amino acid sequence of this Dp cDNA clone shows significant homology with those of Der f 11 and Blo t 11 (9, 12). According to the WHO/IUIS allergen nomenclature subcommittee (13), we propose that this newly identified allergen is designated as Der p 11. In addition, the allergenicity of Der p 11 was also characterized. The recombinant Der p 11 (rDer p 11) showed strong human IgE-binding activities to the sera collected from mite-sensitive asthmatic children.

Materials and methods

Mite extracts

Dry Dp and Df (Allergon Inc., Ängelholm, Sweden) were extracted with 0.01-M cold phosphate-buffered saline, pH 7.2 at a ratio of 1 : 100 (g/ml) as previously described (14). The extract was then dialyzed and freeze-dried for storage at -20°C . The protein content was determined using a Bradford protein assay kit (Bio-Rad, Richmond, CA, USA). Bovine IgG and serum albumin were used as protein standards. However, only live mites were isolated and extracted for mRNA preparation. Breeding colonies of Dp mite was provided by Professor E. L. Hsu (Department and Graduate Institute of Entomology, National Taiwan University, Taipei, Taiwan). Dp mites were bred at 25°C and 75% humidity in 15-cm Petri dishes containing yeast extract and fish powder as culture medium.

Patients' sera

Patients' serum samples were obtained from asthmatic children who attended the Pediatrics Allergy Clinic of Veterans General Hospital-Taipei, Taipei, Taiwan. Fifty mite-sensitive asthmatic children (19 boys and 31 girls) between 4 and 14 years of age were included. All patients had a history of moderate or severe asthmatic symptoms and their sera had IgE-binding reactivity to the crude Dp extract in immunodot and MAST assays (MAST Immunosystems, Inc., Mountain View, CA, USA). Sixteen sera collected from healthy children without IgE-binding reactivity to mite extracts were used as negative controls. The ethical approval to collect sera from children to perform the experiments was permitted by Institutional Review Board, VGH-Taipei (VGHIRB N0. 92-03-06A).

Cloning of Der p 11 cDNA

The Dp cDNA library was prepared as described previously for the preparation of Df cDNA library (9). In brief, 1 g of live Dp mites was harvested to isolate total RNA using RNeasyTM B kit (Qiagen Lab., Inc., Houston, TX, USA). The cDNA library was constructed by a standard method using λ gt11 expression vector (Stratagene, La

Jolla, CA, USA). An anti-Der f 11 mouse monoclonal antibody (mAb642) was used to screen the plaques of the Dp cDNA library (15). Approximately 1×10^6 clones were plated out with Y1090 *Escherichia coli* host on a 150-mm Petri dish containing both media supplemented with 50 $\mu\text{g}/\text{ml}$ of ampicillin. After 6 h of incubation at 37°C , the plate was over-layered with a nitrocellulose filter previously saturated with 10 mM isopropyl β -D-thiogalactopyranoside (IPTG) to induce antigen expression. The cDNAs of positive clones were primed with λ gt11F (5'-TTGACACCAGACCAACTGGT AATG-3') and λ gt11R (5'-GGTGGCGACGACTCCTGGAGCC CG-3') as primers using Taq DNA polymerase. The polymerase chain reaction (PCR) products were purified from 2% agarose gel using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA) and ligated into pCRII-TOPO vector (3.9 kb) using the TOPO TA cloning kit (Invitrogen Co., Groningen, the Netherlands). They were subsequently transformed into the TOP10 competent *E. coli*. The DNAs of plasmids was then analyzed by restriction analysis with *Eco*R1 or by sequencing.

5'-3' RACE reaction

The full-length cDNA of Der p11 was screened by cDNA library immunoscreening, the 5'-3' rapid amplification of cDNA ends system (RACE; Gibco BRL Co., Carlsbad, CA, USA) and PCR. For the 3'-RACE reaction, the cDNA template was synthesized by Moloney murine leukaemia virus (MMLV) reverse transcriptase using 3'-RACE adapter primer (AP, 5'-GGCCACGCGTCGACTAGTACT-(dT)₁₆-3'). The sequence of abridged universal amplification primer (AUAP, 5'-GGCCACGCGTCGACTAGTACT-3') is a part of primer AP. The obtained cDNA template was used for subsequent PCRs: first with primer AUAP and subsequently step-by-step with Der p 11 gene-specific primer 3'-RACE-1 (5'-CAGGCTCTACAAATTACGGA-3'), 3'-RACE-2 (5'-CCGATGAACGAG TACAGAAAC-3') and 3'-RACE-3 (5'-GATCATCGTGTCAAAGAATTGTTG-3'). The PCR products were purified with QIAquick PCR Purification Kit, ligated into pCRII-TOPO vector using the TOPO TA cloning kit and subsequently transformed into TOP10 competent *E. coli*. The plasmid was purified and analyzed by DNA sequencing.

In the 5'-RACE reaction, the cDNA template was synthesized reverse transcriptase MMLV using 1 μg of Dp RNA and gene-specific primer (5'-TTCATC GGCATGTGCTTGC-3') designed according to Der p 11 internal sequence. After the reaction, the RNA was digested with RNaseH and the cDNA was purified by ethanol precipitation. An oligo-(dC) was added to the end of the purified cDNA with terminal deoxynucleotidyl transferase TdT. Primer 5'-RACE-1 (5'-TTCATCGGCATGTGCT TGC-3'), 5'-RACE-2 (5'-TCTTCTTCCAAACGATGGCGAG-3'), 5'-RACE-3 (5'-TGCATTTCTGTGTACGGCAG-3') and 5'-RACE-4 (5'-CACCCTGGATCGGTA CAT-3') designed according to the internal sequence of Der p 11 and 5'-RACE abridged anchor primer (AAP, 5'-GGCCACGCGTCGACTAGTACGGGIIIGG-GIIGGG IIG-3') were used in nested PCR. The PCR product was purified, subcloned, transformed and analyzed as above. The complete nucleotide sequence of Der p 11 was then determined.

Expression of the Der p 11 cDNA clone in *E. coli*

The complete open reading frame encoding Der p 11 was generated by PCR amplification of the cDNAs of Der p 11 with restriction enzymes *Bam*HI and *Sma*I. The PCR product was inserted into the *Bam*HI and *Sma*I sites of the pGEX-4T-1 expression vector (Amersham Pharmacia Biotech Co., Uppsala, Sweden) and

expressed as a glutathione S-transferase (GST) fusion protein in TOP10 competent *E. coli* as previously described (16). Four Der p 11 cDNA fragments (p1, p2, p3 and p4) were similarly generated by PCR amplification. The nucleotide sequences for p1, p2, p3 and p4 were 1–1121, 1098–2628, 1098–1973 and 1972–2625 bp, respectively. These cDNA fragments were also expressed using the GST expression system.

Preparation of recombinant proteins

The recombinant proteins of Der f 11 (rDer f 11), Der p 11 (rDer p 11) and large fragments of Der p 11 (p1–p4) were prepared as described previously (9, 17). In brief, *E. coli* TOP10 containing pGEX-Dp 11 was cultured overnight in Luria broth containing 100 µg/ml of ampicillin to log-phase and then induced with 1 mM of IPTG. After cell harvesting, cell lysate was prepared by sonification. Protein purification was performed by affinity chromatography using a Glutathione Sepharose Column (Sigma-Aldrich, St Louis, MO, USA) according to the protocol described in the GST Purification Kit (Pharmacia Biotech., Piscataway, NJ, USA). Purified proteins were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (18) and by Western blot analysis as previously described (8).

Amino acid sequence analysis

Homologues to the deduced amino acid sequence of Der p 11 were searched through protein and gene databases by using the BLAST program. Sequence analysis and alignment were scanned with the SeqWeb Version 1.2 (Pharmacia Inc., San Diego, CA, USA) and the Wisconsin Package Version 10.1 (Genetics Computer Groups Inc., San Diego, CA, USA).

SDS-PAGE and immunoblot

Immunoblotting was performed as previously described (19). Briefly, rDer p 11, fragments p1–p4, rDer f 11 (3 µg/lane) and Dp crude extract (5 µg/lane) first separated in 12.5% SDS-PAGE were electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Bedford, MA, USA) and then blotted with serum samples. After blocking overnight with 5% skimmed milk, blots were immersed with pooled human serum (1 : 2) overnight at 4°C. Thereafter, they were immersed with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgE antibodies (1 : 2500; DAKO Corp., Carpinteria, CA, USA) at room temperature (RT) for 5 h. Blots were washed with Tris-buffered saline (TBS) after each treatment. At last, blots were immersed with ECL reagent (Amersham, Buckinghamshire, UK) for 2 min and exposed to X-ray films for 5–30 s at RT. In order to assess specific antibody-binding capacity, an immunoblot was immersed with mAb642 (1 : 10 000) for 1 h at RT. After washing, the blot was immersed with HRP-conjugated rabbit anti-mouse IgG (1 : 3000; DAKO) for 1 h at RT. The following steps were described as above.

2D-gel electrophoresis

The 2D-gel electrophoresis was performed as described previously (8). In brief, isoelectric focusing in the first dimension was carried out in 3.5% polyacrylamide gels in glass tubes. The gels were then extruded from the glass tubes and equilibrated in TBS containing glycerol (10%), SDS (2.1%) and bromophenol blue (pH 6.8). The second dimension of electrophoresis was carried out on slab gel consisting of 13% polyacrylamide in Protean II 2D cell (Bio-Rad

Lab., Hercules, CA, USA). High MW standards were used as a reference. Carbamylated creatine standard (BDH, Poole, UK) was used as the charge standard for calculation of isoelectric point values of the resolved proteins.

Immunodot analysis for human-specific IgE-binding capacity

The immunodot assay for human-specific IgE-binding reactivity was performed as previously described (19). Briefly, crude Dp extract (3 µg/dot) and recombinant proteins (rDer p 11, fragments p1–p4) (2 µg/dot) were spotted on a PVDF membrane. Whereas crude Dp extract, rDer f 11 and rDer p 2 (a gift from Dr C.K. Chua, University of Singapore, Singapore) were used as positive controls, GST was used as a negative control. The subsequent procedures for immunostaining of dot blots were performed as described in the immunoblot assay. The intensity of human IgE-reacting dot was determined by transmission densitometer (ImageQuant Analyzer; Molecular Dynamics Inc., Sunnyvale, CA, USA) with ImageQuant software according to the user's guide. The mean of IgE-reacting intensity from negative controls plus 2 SD was used as a cut-off value for positive reactions.

Inhibition immunoblot tests

Inhibition immunoblot tests were used to determine the cross-reactivity between rDer p 11 and rDer f 11 or the crude mite extract. The pooled mite-allergic serum was first absorbed respectively at 4°C overnight with different concentrations (0, 1 or 100 µg/ml) of rDer p 11 or rDer f 11 pre-coated onto the PVDF membrane. The crude Dp or Df extract (5 µg/lane), rDer p 11 and rDer f 11 (3 µg/lane) were separated by SDS-PAGE and transferred onto PVDF membranes. The membrane strips were then immersed with the pre-absorbed allergic serum respectively. The subsequent procedures were performed as described in the immunoblot assay. The intensity of human IgE-binding reactions was determined by the transmission densitometer.

Results

Isolation of the cDNA clone and cDNA sequence analysis

Twenty-two positive plaques were obtained after screening approximately 1×10^6 plaque-forming units from the Dp cDNA library with mAb642. Five positive clones were isolated and sequenced. Nucleotide sequence analysis showed that these clones were highly homogenous except a different degree of truncations on 5' and 3' ends. The nucleotide sequence alignment of these clones exhibited significant homology to Der f 11. Clone Dp23, the longest one, comprises 1215 bp (from sequence 897 to 2111; data not shown). The full-length Der p 11 cDNA clone was isolated by PCR using gene-specific primers based on the nucleotide sequence of truncated clone Dp23 and 5'- and 3'-RACE products. Der p 11 is a 2965-bp cDNA gene with a 2625-bp open reading frame encoding a protein with 875 amino acid residues. The ATG codon is located at nucleotide positions 182–184 bp. No further upstream N-terminal ATG codon can be found. A typical polyadenylated site TAA at nucleotide positions 2807–2809, the C-terminal of the

Der p 11	1***** MSARTAKMY	ηηηηη RSSGAGASGD	ISVEYGTDLG	**** ALTRLEDKIR	LLSDDLSESR	*** EMRQRIEREK	AELQIQVMSL	70
Der f 11	~~~~~ ~~~~~	~~~~~ ~~~~~	~~~~~ ~~~~~	~~~~~ ~~~~~	~~~~~ ~~~~~	~~~~~ ~~~~~	~~~~~ ~~~~~	
Blo t 11	-a--s-----	q--r--hg--	--i-----	-----	---e-----	-l---v-----	sdlitv-l-n-	
Der p 11	71*** SERLEEAEGS	**** SESVTEMNKK	*** RDSELAKLK	LLEDVHMESE	ETAHHLRQKH	QAAVHEMQDQ	LDQLQKAKNK	140
Der f 11	~~~~~ ~~~~~	~~~~~ ~~~~~	~~~~~ ~~~~~	-----i--	-----	---iq-----	-----	
Blo t 11	t-----t--	-----	-----	-----	-----	-----	---v-----	
Der p 11	141*** SDKEKQKFQA	**** EVFELLSQLE	TANKEKLTA	***** KSVEKLEYTV	** HELNIKIEEI	**** NRTVIELTSH	* KQRLTQENTE	210
Der f 11	-----	-----a--	-----	-n-----	-----	-----	---s-----	
Blo t 11	-----	-----a-v--	-----d--v-q	-t-----	-----	---v-v-a--	r---s-----s-	
Der p 11	211 LIKEVHEVKL	QLDNANHLKQ	QIAQQLEDTR	HRLEEEERKR	**** SSLENHAHTL	*** EVELESKVKQ	LDDESEARLE	280
Der f 11	-----	-----t--	-----	-----	a-----	-----	---e-----	
Blo t 11	-----y-i	s-----g	-----	-----d--	-----	-----	---ee-----	
Der p 11	281 LERQLTKANG	*** DAASWKSQYE	AELQAHADDEV	EELRRKMAQK	**** ISEYEEQLEA	**** LLNKCSSLK	**** QKSRLQSEVE	350
Der f 11	-----	-----	-----	-----	-----	-----	-----	
Blo t 11	-----	-----	-----v--	-----	---g-----	---a-----	---a-----	
Der p 11	351 VLIMDLEKAA	AHAQQLEKRV	AQLEKINLDL	KDKLEEVMTL	MEQAQKELRI	KIGELQKLQH	EYEKVRDQRD	420
Der f 11	-----t	r-----	-----	-n-----	-----v--	---a-----	---l-----	
Blo t 11	-----t	---a-----	s-----	-s-----s-	l--t--d--v	--ad-----	---l--ke	
Der p 11	421 QLARENKKLT	DDLAEAKSQL	NDARRRIHEQ	EIEIKRLNE	RDELSAAYKE	**** AETLRKQEEA	KNQRLIAELA	490
Der f 11	-----	-----	-----	-----	-----	-----	-----	
Blo t 11	a-----a	-----	-----	-----	-e-a-----	-----	---t-----	
Der p 11	491 QVRHDYKRL	AQKDEEIEAL	RKQYQIEIEQ	LNMLAEAEA	KLKTEIARLK	*** KKYQAQITEL	* ELSLDAANKA	560
Der f 11	-----	-----	-----	-----	-----	-----	-----	
Blo t 11	-t-----	---e-----	-----	-----	---v-----	-----	-----	
Der p 11	561 NIDLQKTIKK	QALQITELQA	HYDEVHRQLQ	QAVDQLGVTQ	** RRCQALQAL	EEMRIALEQA	SRAKRQAEQL	630
Der f 11	-----	-----	-----	-----	-----	-----	n-----	
Blo t 11	-----	---g-----	-----	-----	---t-----	---vn-----	l---a---m	
Der p 11	631 HEEAVVRVNE	LTTINVLAS	AKSKLESEFS	ALQADYDEVH	KELRISDERV	QKLTIELKST	** KDLLIEEQER	700
Der f 11	-----	-----	-----	-----	-----	-----	-----	
Blo t 11	-----	-----	-----t--	-n-----	-----	---v-----	---es-t--	
Der p 11	701 LVKLETVKKS	LEQEVRTLHV	RIEEVEANAL	φφφφ AGGKRVIACL	ESRIRDVEIE	VEEERRRHAE	*** TEKMLRKGDH	770
Der f 11	-----	-----	-----	-----	-----	-----	-d-----	
Blo t 11	vt---i---	--t---n-qi	-----	-----	-----	-----	-----	
Der p 11	771 RVKELLQNE	EDHKQIQLLQ	*** EMTDKLNKQV	KVYKRQMGEQ	EGMSQQ NLTR	VRRFQRELEA	AEDRADQAES	840
Der f 11	-----	-----	-----	-----	-----	-----	-----	
Blo t 11	-----	-----	---s-----	-----	-----	-----	-----	
Der p 11	841 NLSF IRAKHR	SWVTTSQVPG	**** GTRQVFTTQE	ETTN* ETTN*	-----	-----	-----	875
Der f 11	-----	-----	-----	-----	-----	-----	-----	
Blo t 11	-----	-----	-----v-eq	-ss-f*	-----	-----	-----	

Figure 1. The amino acid sequence of Der p 11, Der f 11 and Blo t 11. The amino acid residues are numbered on the right and left, respectively. The four predicted N-linked glycosylation sites are shown in bold italicized letters while the predicted leucine zipper motifs are single underlined. The predicted glycosaminoglycan site (η), phosphorylation sites (*) and amidation site (ϕ) are indicated. Identical residues are indicated as dashed lines. The full-length sequence of Der p 11 is longer than the truncated sequence of Der f 11 and shown as symbol (~) on both termini of the sequence. The sequence information of Der p 11 and Der f 11 is available on the GenBank sequence database under the accession number AY189697 and AF352244, respectively.

cDNA insert, suggests that Der p 11 is a full-length cDNA clone (the accession number AY189697 in the GenBank sequence database).

Der p 11 shares high sequence homology with paramyosin of invertebrates

The nucleotide sequence and deduced amino acid sequence of Der p 11 were compared with others in

Swiss-Prot and GeneBank databases using BLAST. Both sequence analysis and alignment were performed using SeqWeb Version packages. The homology research revealed that this high-MW Dp allergen had high degrees of sequence identity with paramyosin of invertebrates. Amino acid sequence alignment showed that Der p 11 shared 89% and 97% identity with Blo t 11 and truncated Der f 11, respectively (Fig. 1). The amino acid composition of Der p 11, Der f 11 and Blo t 11 all have some

Table 1. Frequency of IgE-binding reactivity of allergic sera to crude Dp extract, rDer p 11, rDer f 11, GST-rDer p 2 and GST in a panel of 20 Dp mite-extract positive sera collected from asthmatic children and seven control sera collected from healthy children was analyzed by dot-blot immunoassay

	Mite components				GST (control)
	Crude Dp	rDer p 11	rDer f 11	rDer p 2	
IgE reactivity (positive %)					
Asthmatics	20 (100)	15 (75)	13 (65)	15 (75)	1 (5)
Healthy controls	0	0	0	0	0

common characteristics with the paramyosin of invertebrates. They all have high percentage (about 41%) of charged residues such as D, E, H, K and R (Table 1). Similar to Der f 11 and Blo t 11, Der p 11 has several relatively long leucine zipper motifs (Fig. 1). These motifs may play an important role in the folding of protein structure.

Recombinant Der p 11 and its polypeptide fragments

A cDNA fragment containing *Bam*HI restriction enzyme cutting sites was PCR-amplified from Der p 11 cDNAs and subcloned into the pGEX-4T-1 expression vector. Recombinant Der p 11 (rDer p 11) was expressed in the *E. coli* expression system as a fusion protein with GST and analyzed using SDS-PAGE. The MWs of rDer p 11-GST, rDer p 11 (without GST) and rDer f 11 (a control protein) are approximately 129, 103 and 94 kDa, respectively (Fig. 2A). The MWs of Der p 11 fragments p1, p2, p3 and p4 with GST (26 kDa) were approximately 72, 82, 61 and 45 kDa, respectively (Fig. 2B). Using 2D-electrophoresis, mAb642-recognized rDer p 11 (without GST) has the MW of about 103 kDa and isoelectric point value at 5.8 (arrow pointed, Fig. 2C).

Human IgE antibody-binding capacity of rDer p 11

Both immunoblot (A) and immunodot (B) analyses were first performed and revealed that mAb642 bound specifically to rDer p 11-GST, p2 and p3 (Fig. 3). Secondly, the allergenicity of rDer p11-GST and its fragments p1, p2, p3 and p4 was further determined by their capacity to react with pooled allergic human sera. Using immunoblotting, human IgE-binding reactivity was merely found in rDer p 11-GST and its fragments, but not in GST (Fig. 4). Multiple human IgE-binding activities distributed over four fragments of Der p 11 suggest that there are several human IgE-binding determinants in rDer p 11.

Thirdly, the intensity of IgE-binding reactivity to each individual mite-allergic patient or healthy volunteer was assessed quantitatively using the transmission-densitometer. The estimated cut-off values (mean $\pm$ 2 SD) of IgE-binding capacity among 16 healthy children was 625.3 (509.5 $\pm$ 17.4) for Dp crude extract, 737.3

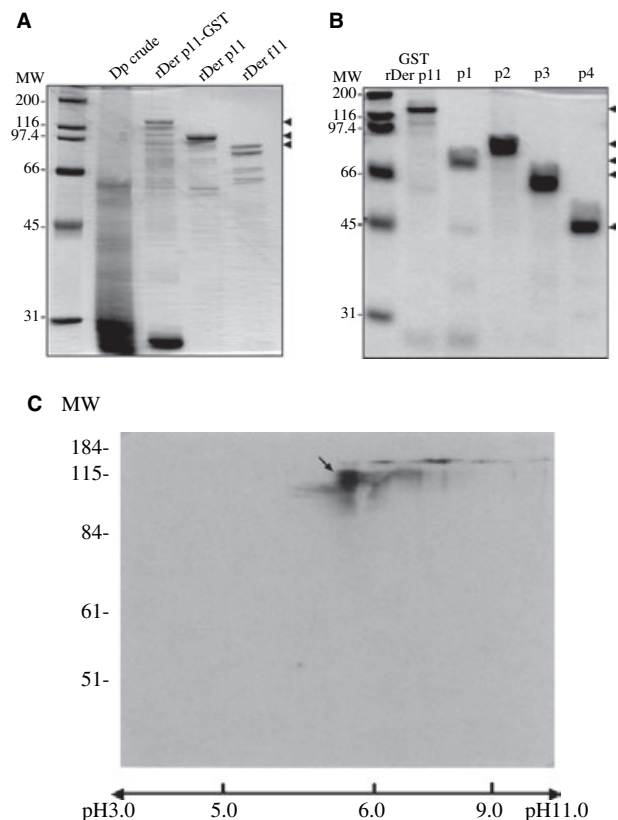


Figure 2. SDS-PAGE and immunoblot (9, 12of rDer p 11. Recombinant proteins (3 μ g/lane) and crude Dp mite extract (5 μ g/lane) were analyzed by SDS-PAGE and then visualized by staining with Coomassie brilliant blue. (A) SDS-PAGE of a fusion protein with GST (rDer p 11-GST) and without GST (rDer p 11 or rDer f 11). (B) SDS-PAGE of rDer p 11-GST and recombinant fragments p1 to p4. (C) After 2D-electrophoresis and immunoblotting, the MW and isoelectric point value of mAb642 antibody-recognized rDer p 11 was shown here (arrow pointed).

(641.4 $\pm$ 47.8) for rDer p 11, 618 (591.2 $\pm$ 13.4) for rDer f 11 and 585.7 (574.3 $\pm$ 5.7) for control GST. The results of the IgE-binding immunodot assay for 20 Dp extract-positive asthmatic children and seven healthy children are summarized in Table 1. The results show 75% (15/20), 65% (13/20), 75% (15/20) and 5% (1/20) of positive IgE-binding reactivity to rDer p 11, rDer f 11, GST-rDer p 2 and GST, respectively. Whereas GST was used as a negative control, the rDer f 11 and GST-rDer p 2 were included as positive controls (Fig. 5A,B).

Finally, 30 Dp extract-positive serum samples were included in another panel of immunodot assays for human IgE-binding reactivity to rDer p 11 and its four fragments (Fig. 6A,B). The human IgE-binding reactivity to rDer p 11, p1, p2, p3, p4 and GST were 80% (24/30), 46.7% (14/30), 73.3% (22/30), 66.7% (20/30), 50% (15/30) and 6.6% (2/30), respectively. No positive IgE reactivity to rDer p 11 and its fragments was found in

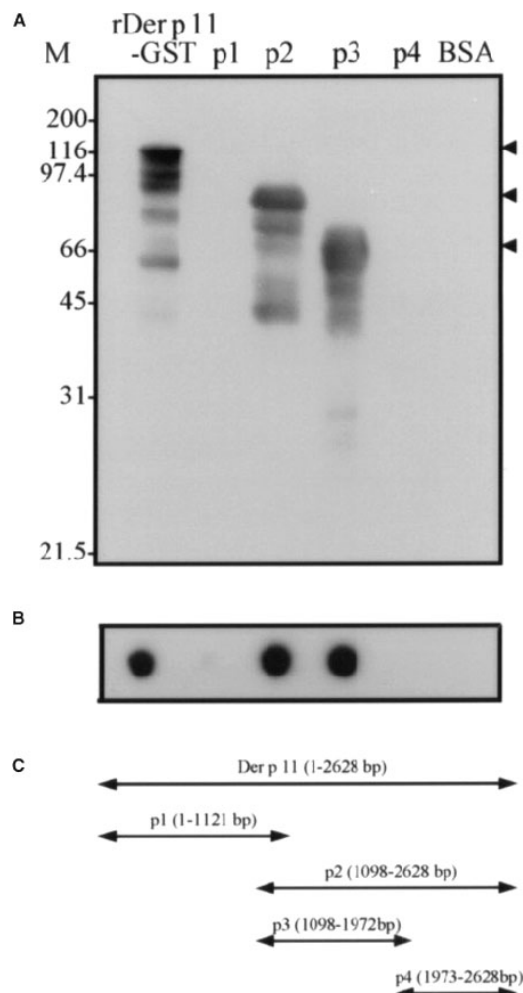


Figure 3. Immunoblot (A) and immunodot (B) analyses were performed to examine the specific reactivity of mAb642 to recombinant fragments p1 to p4 and rDer p 11-GST. All recombinant proteins (3 µg/lane) were separated by 12% SDS-polyacrylamide gel and then electroblotted on PVDF membrane or dotted directly (2 µg/dot) on membrane. The blots were probed subsequently with mAb642 (diluted 1 : 10 000), HRP-conjugated rabbit anti-mouse IgG (1 : 3000) and ECL reagent. (C) Schematic diagram shows strategy for generation of Der p 11-derived fragments by PCR. Numbers indicate nucleotide sequence of cDNA insert; p1 to p4 represent four peptide fragments.

nine healthy control serum samples. When the last two studies were included, 78% (39/50) of Dp extract-positive sera collected from asthmatic children had specific IgE-binding reactivity to rDer p 11.

Cross-reactivity between rDer p 11 and rDer f 11 or the crude mite extract

IgE-binding cross-reactivity between rDer p 11 and rDer f 11 or the crude mite extract was performed using inhibition immunoblot tests (Fig. 7). IgE-binding reactiv-

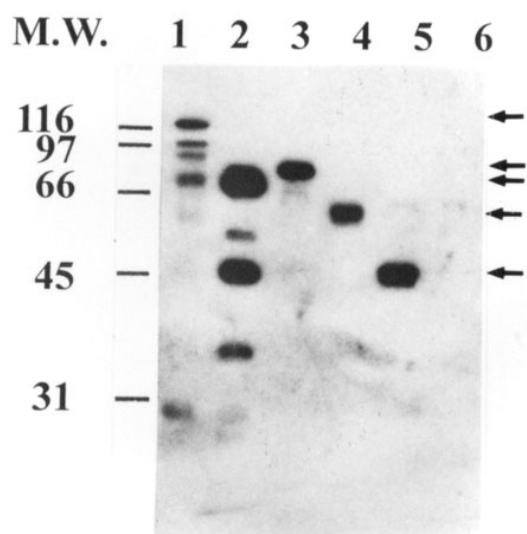


Figure 4. Human IgE-binding reactivity to rDer p 11-GST and the recombinant peptide fragments p1 to p4 using immunoblotting. All recombinant proteins (3 µg/lane) were separated by SDS-PAGE and transferred onto PVDF membranes. The membrane strips were probed with the pooled allergic serum (1 : 2 diluted). The blots were subsequently probed with HRP-conjugated rabbit anti-human IgE antibodies (1 : 2500 diluted) and ECL reagent.

ity to Der p 11 in the crude Dp extract was inhibited in the pooled allergic serum pre-absorbed with 1 and 100 µg/ml of rDer p 11 (53 and 78%, respectively; Fig. 7A). Similarly, IgE-binding reactivity to Der f 11 in the crude Df extract was inhibited in the pooled allergic serum pre-absorbed with 1 and 100 µg/ml of rDer f 11 (58 and 83%, respectively; Fig. 7B). However, IgE-binding reactivity to rDer p 11 was inhibited in the pooled allergic serum pre-absorbed with 1 and 100 µg/ml of rDer p 11 (66 and 95%, respectively) and rDer f 11 (47 and 80%, respectively) (Fig. 7A). Similarly, IgE-binding reactivity to rDer f 11 was inhibited in the pooled allergic serum pre-absorbed with 1 and 100 µg/ml of rDer f 11 (42 and 68%, respectively) and rDer p 11 (15 and 73%, respectively) (Fig. 7B).

Discussion

In the present study, we have cloned for the first time the full-length cDNA clone encoding a novel high-MW mite allergen Der p 11 from the Dp cDNA library using immunoscreening, the 5'- and 3'-RACE system and PCR. This newly identified Der p 11 clone encodes a large mite allergen with 875 amino acid residues and with the MW up to 103 kDa. We have demonstrated that the amino acid sequence deduced from this novel Dp cDNA clone possessed significant homology with the paramyosin of different invertebrates (20, 21). In amino acid sequence analysis and alignment, the deduced polypeptide of Der p 11 shares over 89% identity with Der f 11 or Blo t 11

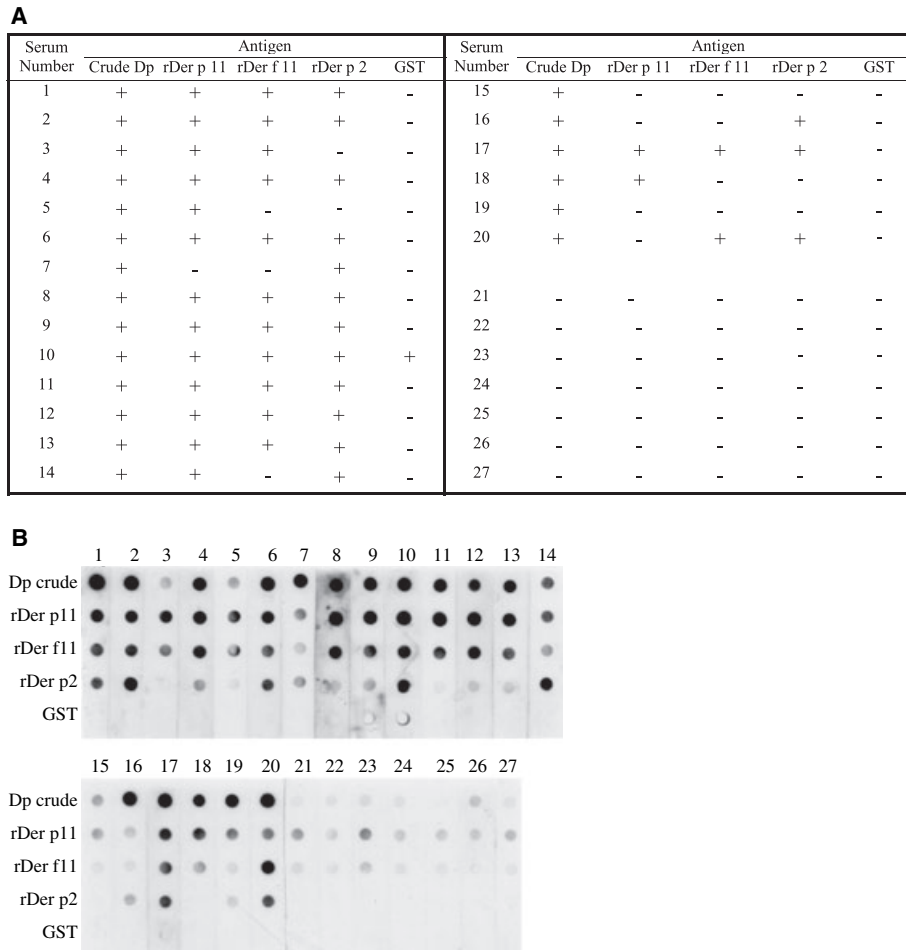


Figure 5. Allergenicity of rDer p 11 and rDer f 11. (A) Human IgE-binding reactivity to rDer p 11, rDer f 11, GST-rDer p 2 and control GST protein among 20 Dp extract-positive sera (no. 1 to 20) and seven control normal sera (no. 21 to 27) is summarized. Reactivity was considered positive if the intensity of the tested sera were higher than cut-off value (mean + 2 SD) derived from healthy controls. Positive (+) and negative (-) reactions are shown. (B) The immunodot assay of human IgE-binding reactivity for 20 Dp extract-positive sera and seven normal sera is shown.

(9, 12). The similarity in their amino acid sequence suggests that Der f 11, Der p 11 and Blo t 11 may be evolved originally from the same ancestor gene. Mouse monoclonal antibody mAb642 initially cloned from mice immunized with Der f 11 (8, 9) was successfully employed for immunoscreening of the Der p 11 cDNA clone from the Dp cDNA library. In the subsequent immunoblot and immunodot studies, mAb642 also showed strong reactivity to Der p 11 (Fig. 3A,B). This finding indicates that Der p 11 and Der f 11 may have a common antibody-binding determinant, or two highly similar antigenic determinants both with binding reactivity to mAb642. Consistent with previous reports that the allergens derived from closely related species may preserve a high degree of human IgE cross-reactivity (22, 23), we did confirm here that both Der p 11 and Der f 11 had strong IgE-binding reactivity to the sera collected from mite-allergic asthmatic children (Fig. 5). Furthermore, the

cross-inhibiting reactivity between rDer p 11 and native Der p 11 in the crude extract was first confirmed by the inhibition immunoblot assay (Fig. 7A). The cross-inhibition between rDer p 11 and Dp crude extract suggests that rDer p 11 and native Der p 11 in the crude extract have the same IgE-binding epitopes. In addition, cross-inhibition effects between rDer p 11 and rDer f 11 were also confirmed by the inhibition immunoblot assay (Fig. 7). These cross-inhibition experiments suggest that rDer p 11 and rDer f 11 share cross-reactive human IgE-binding epitopes. The present results indicate that, similar to rDer f 11, rDer p 11 is also a crucial allergen in the diagnostic and therapeutic application of mite allergy. Since the immunological and biochemical characteristics of this 103 kDa Dp mite protein allergen are highly similar to those of Der f 11, it is therefore denominated as Der p 11 according to the WHO/IUIS allergen nomenclature subcommittee (13). Our recent study has further

A

Serum		Antigen						Serum		Antigen					
number	rDerp11	p1	p2	p3	p4	GST		number	rDerp11	p1	p2	p3	p4	GST	
P1	+	+	+	+	+	-		P21	+	+	+	-	-	-	
P2	+	-	+	+	+	-		P22	+	+	+	+	+	-	
P3	+	-	+	+	-	-		P23	+	+	+	+	+	-	
P4	+	+	+	+	+	-		P24	+	+	+	+	+	+	
P5	+	+	+	+	+	-		P25	+	+	+	-	-	-	
P6	+	-	-	-	-	-		P26	+	-	-	-	-	-	
P7	-	-	-	-	-	-		P27	-	-	-	-	-	-	
P8	+	+	+	+	+	-		P28	-	-	+	-	+	-	
P9	+	+	+	-	-	-		P29	-	-	-	-	-	-	
P10	-	-	-	-	-	-		P30	-	-	-	-	-	-	
P11	+	+	+	+	+	-		N1	-	-	-	-	-	-	
P12	+	-	+	+	-	-		N2	-	-	-	-	-	-	
P13	+	+	+	+	+	-		N3	-	-	-	-	-	-	
P14	+	+	+	+	-	-		N4	-	-	-	-	-	-	
P15	+	+	+	+	+	-		N5	-	-	-	-	-	-	
P16	+	+	+	+	+	-		N6	-	-	-	-	-	-	
P17	+	+	+	+	+	+		N7	-	-	-	-	-	-	
P18	+	-	+	+	+	-		N8	-	-	-	-	-	-	
P19	-	-	-	-	-	-		N9	-	-	-	-	-	-	
P20	+	-	-	+	-	-									

Total number of rDer p11, p1, p2, p3, p4 and GST is 24, 14, 22, 20, 15 and 2 in 30 Dp extract-positive asthmatic sera (P) and 9 normal control sera (N) in dot blot analysis

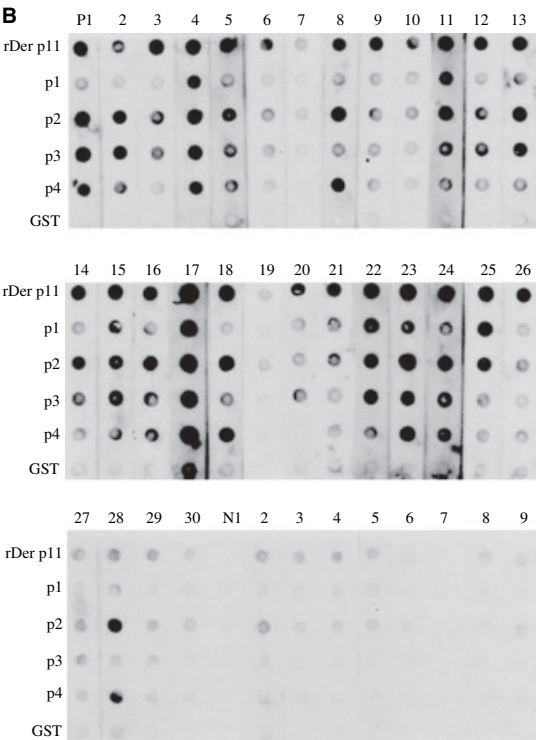


Figure 6. Allergenicity of rDer p 11 and its fragments p1 to p4. (A) Human IgE-binding reactivity to rDer p 11, p1, p2, p3, p4 and the control GST protein among 30 Dp extract-positive sera (P1 to 30) and nine normal control sera (N1 to N9) is summarized. (B) The immunoblot assay of human IgE-binding reactivity for 30 Dp extract-positive sera and nine normal sera.

shown that T cells collected from the asthmatic children with positive CAP reactions have much stronger T-cell proliferation responses to Dp extracts than those healthy ones with negative CAP reactions to Dp extracts (C. Y. Lin and L. C. Tsai, unpublished data).

The amino-acid composition of Der p 11 and Der f 11 also shares a number of other common characteristics. For instance, they both contain high proportions of charged residues (Asp, Glu, Lys and Arg) (38–40%), Asn plus Gln (12%), hydrophobic residues Leu and Ala

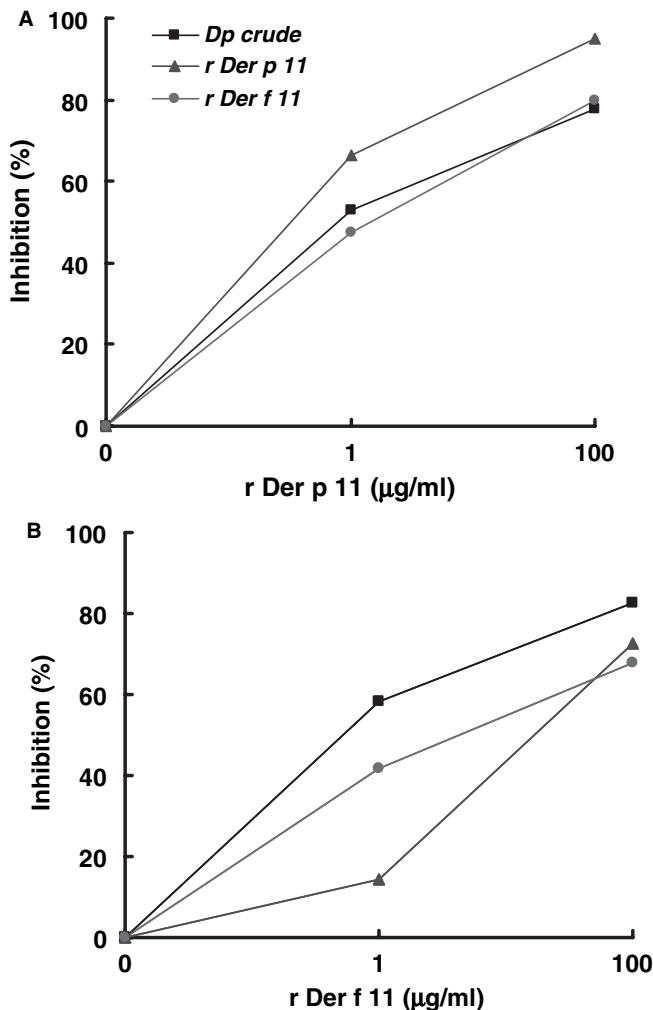


Figure 7. Cross-inhibition reactions were performed by the inhibition immunoblot tests. The crude Dp or Df extract, rDer p 11 and rDer f 11 were separated by SDS-PAGE and transferred onto PVDF membranes. The membrane strips were then probed with the pooled allergic serum that was pre-absorbed with 0, 1 or 100 μg of rDer p 11 (A) or rDer f 11 (B) overnight at 4°C.

(12–14% and 8–11%, respectively), but low contents of Cys, Phe, Pro, Trp (<1%) and absence of Asx and Glx. Like the paramyosin of other invertebrates, leucine (12.5%) is the most abundant hydrophobic residue in amino acid contents of Der p 11. Several relatively long leucine zipper motifs were previously reported in Der f 11 (9). They were also found in Der p 11. It is possible that they may play an important role in the folding of paramyosin. Another common characteristic feature of paramyosin is the presence of a serine-rich N-terminal region (24, 25). Der p 11 contains 5.257% of serine residues (Fig. 1). We have shown that Der f 11 contains 3.8% of serine residues (9). The difference between two reports might be due to truncation of sequence of Der f 11. Two putative N-glycosylation sites have been described previously for Der f 11 (9). Four putative

N-glycosylation sites were identified recently in Blo t 11 at residues number 139–142, 191–194, 817–820 and 841–844 (12) and here in Der p 11 at residues number 139–142, 191–194, 817–820 and 841–844 (Fig. 1). These N-glycosylation sites are probably functional because high mannose N-glycan chains have been found in the native form of Der f 11 (8). In contrast to truncation of Der f 11 at both N- and C-termini (9), the full-length nucleotide sequence of Der p 11 consists of nucleotides with an initiation codon (ATG at nucleotide positions 1–3) and a termination codon (TAA at nucleotide positions 2626–2628) (the accession number AY189697 in the GenBank sequence database).

Consistent with our report that rDer f 11 was readily degraded during preparation (8, 9, 19), rDer p 11 was also partially degraded into a number of smaller GST-containing fragments with MW over 45 kDa (Fig. 2A). In order to further evaluate potential human IgE-binding determinants in rDer p 11, four recombinant polypeptide fragments of Der p 11 (p1 to p4) were generated here and used to examine their human IgE-binding capacity using the immunodot assay (Fig. 6). Interestingly, rDer p 11 and its degraded fragments all could bind to mAb642 (Fig. 3A,B). This finding reveals that the mAb642-binding determinant must be present in fragment p3 (Fig. 3A,B). Among 30 Dp mite-positive sera, over 50% sera showed human IgE-binding reactivity to all four polypeptide fragments (Fig. 6). This result implies that, for about half of Dp-allergic patients, several human IgE-binding sites are scattered throughout the entire molecule of Der p 11. Besides, 73% and 66% of Dp mite-positive serum samples reacted positively with p2 and p3 fragment, respectively (Fig. 6). These data imply that, for more than half of Dp-allergic patients, polypeptide fragment p2 and p3 may individually contain at least one human IgE-binding determinant. Furthermore, smaller polypeptide fragments of Der p 11 are needed for further mapping major human IgE-binding epitopes in the future study.

Up to date, 19 groups of mite allergens have been identified. Among them, major human IgE-binding reactivity has been reported for the group 1, 2, 3, 9, 11 and 14 allergens (3). High MW group 11 and 14 mite allergens have been recently described (8–10, 12). Among 50 Dp extract-positive sera, rDer p 11 shows a higher frequency of human IgE-binding reactivity (78%; 39/50 in Figs 5 and 6). Its prevalence is higher than that of rDer f 11 (65%; 13/20 in Table 1). The frequency of its human IgE-binding reactivity is similar to that of major group 2 mite allergen (Table 1). Therefore, it can be concluded that, similar to Der f 11, Der p 11 is also an important and major mite allergen. The clinical importance of high MW mite allergens in asthmatic patients has been described previously for Der f 11 (8, 9, 19, 26) and presently for Der p 11. However, Der p 11 and Der f 11 are both present as minor protein components in the crude extract of Dp and Df mites (Fig. 2A) (8, 9). They are hardly to be

purified up to the potential scale for allergen-specific immunotherapy. Therefore, preparation of recombinant proteins seems to offer an easy but unique way to get good substitutes. To sum up, the isolation and identification of Der p 11 is an important step to further characterize the complete spectrum of its human IgE-binding reactivity in house dust mite allergens (27), and to design its potential peptide vaccines without triggering allergic reactions (28, 29).

Acknowledgments

We are grateful to Ms H. Y. Lai for her technical assistance. We are also grateful to Dr K.-Y. Chua, Department of Paediatrics, Faculty of Medicine, National University of Singapore, Singapore, for her advice in sequencing techniques. This study was supported by grants VGH88-384 from Taipei-Veterans General Hospital and from the National Science Council, Taipei, Taiwan.

References

- Platts-Mills TAE, Rakes G, Heymann PW. The relevance of allergen exposure to the development of asthma in childhood. *J Allergy Clin Immunol* 2000;**105**:S503–S508.
- Tovey ER, Baldo BA. Comparison by electroblotting of IgE-binding components in extracts of house dust mite bodies and spent mite culture. *J Allergy Clin Immunol* 1987;**79**:93–102.
- Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brieh RM. Characterization and immunobiology of house dust mite allergens. *Int Arch Allergy Immunol* 2002;**129**:1–18.
- Stewart GA, Thompson OJ. The biochemistry of common aeroallergens. *Clin Exp Allergy* 1996;**26**:1020–1044.
- Thomas W. Mite allergens group I-VII. A catalogue of enzymes. *Clin Exp Allergy* 1993;**23**:350–353.
- Lin KL, Wang SY, Hsieh KH. Analysis of house dust mite-specific IgE, IgG4 and IgG antibodies during immunotherapy in asthmatic children. *Ann Allergy* 1991;**67**:63–69.
- Tang RB, Tsai LC, Hung MW, Hwang B, Wu KG. Detection of house dust mite allergens and immunoblot analysis in asthmatic children. *J Asthma* 1988;**25**:83–88.
- Tsai LC, Chao PL, Shen HD, Tang RB, Chang TC, Chang ZN et al. Isolation and characterization of a novel 98-kd *Dermatophagoides farinae* mite allergen. *J Allergy Clin Immunol* 1998;**102**:295–303.
- Tsai LC, Sun YC, Chao PL, Ng HP, Hung MW, Hsieh KH et al. Sequence analysis and expression of a cDNA clone encoding a 98-kd allergen in *Dermatophagoides farinae*. *Clin Exp Allergy* 1999;**29**:1606–1613.
- Fujikawa A, Uchida K, Yanagidani A, Kawamoto S, Aki T, Shigeta S et al. Altered antigenicity of M-177, a 177-kDa allergen from the house dust mite *Dermatophagoides farinae*, in stored extract. *Clin Exp Allergy* 1998;**28**:1549–1558.
- McCall C, Hunter S, Stedman K, Weber E, Hillier A, Bozic C et al. Characterization and cloning of a major high molecular weight house dust mite allergen (Der f 15) for dogs. *Vet Immunol Immunopathol* 2001;**78**:231–247.
- John Donnie A R, Cheong N, Lee BW, Chua KY. CDNA cloning and expression of Blo t 11, the *Blomia tropicalis* allergen homologous to paramyosin. *Int Arch Allergy Immunol* 2001;**126**:286–293.
- Hoffman D, Lowenwtein H, Marsh DG, Platts-Mills TA, Thoma WR. Allergen nomenclature. WHO/IUIS Allergen Nomenclature Subcommittee. *Int Arch Allergy Immunol* 1994;**105**:224–233.
- Tang RB, Tsai LC, Hwang HM, Hwang B, Wu KG, Hung MW. The prevalence of allergic disease and IgE antibodies to house dust mite in school-children in Taiwan. *Clin Exp Allergy* 1990;**20**:33–38.
- Chua KY, Doyle CR, Simpson RJ, Turker KJ, Stewart GA, Thomas WR. Isolation of cDNA coding for the major mite allergen Der p 2 by IgE plaque immunoassay. *Int Arch Allergy Appl Immunol* 1990;**91**:118–123.
- Smith DB, Johnson KS. Single-step purification of polypeptides expressed in *Escherichia coli* as fusion with glutathione S-transferase. *Gene* 1988;**67**:32–40.
- Chua KY, Greene WK, Kehal PK, Thomas WR. IgE binding studies with large peptides expressed from Der p 2 cDNA constructs. *Clin Exp Allergy* 1991;**21**:161–166.
- Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–681.
- Tsai LC, Sun YC, Chao PL, Hung MW, Kuo IC, Liaw SH et al. Protein sequence analysis and mapping of IgE and IgG epitopes of an allergenic 98 kda *Dermatophagoides farinae* paramyosin, Der f 11. *Allergy* 2000;**55**:141–147.
- Landa A, Lacleite JP, Shoemaker CB. CDNA cloning and recombinant expression of collagen-binding and complement inhibitor activity of *Taenia solium* paramyosin (AgB). *Mol Biochem Parasitol* 1993;**60**:343–347.
- Matsumoto Y, Perry G, Levine RJC, Blanton R, Mahmoud AAF, Aikawa W. Paramyosin and actin in schistosomal teguments. *Nature* 1998;**333**:76–78.
- Tsai LC, Tang RB, Hung MW, Chen HM, Hwang LA. Correlation between serum IgE and specific IgE antibody titer to house dust mite in children with asthma. *J Asthma* 1988;**25**:7–13.
- Arlian LG, Bernstein IL, Vyszynski-Mohr DL, Gallagher JS. Investigations of culture medium-free house dust mites. IV. Cross antigenicity and allergenicity between the house dust mites, *Dermatophagoides farinae* and *D. pteronyssinus*. *J Allergy Clin Immunol* 1987;**79**:467–477.
- Ramos JDA, Teo ASM, Ou KL, Tsai LC, Lee BW, Cheong N et al. Comparative allergenicity studies of native and recombinant *Blomia tropicalis* paramyosin (Blo t 11). *Allergy* 2003;**58**:412–419.
- Schriefer LG, Waterson RH. Phosphorylation of the N-terminal region of *Caenorhabditis elegans* paramyosin. *J Mol Biol* 1989;**207**:451–454.
- Chao PL, Peng HJ, Tang RB, Hung MW, Tsai LC. Serum specific IgE reactivity to recombinant Der f 11 in asthmatic children. *J Asthma* 2001;**38**:391–398.

27. Thomas WR, Smith W. Towards defining the full spectrum of important house dust mite allergens. *Clin Exp Allergy* 1999;**29**:1583–1587.
28. Peng HJ, Su SN, Chang ZN, Chao PL, Kuo SW, Tsai LC. Induction of specific Th1 responses and suppression of IgE antibody formation by vaccination with plasmid DNA encoding Der f 11. *Vaccine* 2002;**20**:1761–1768.
29. Peng HJ, Tsai LC, Su SN, Chang ZN, Shen HD, Chao PL et al. Comparison of different adjuvants of protein and DNA vaccination for the prophylaxis of IgE antibody formation. *Vaccine* 2004;**22**:755–761.