cDNA cloning, sequence analysis and allergological characterization of Par j 2.0101, a new major allergen of the *Parietaria judaica* pollen

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Abstract A clone (P2) coding for an allergen of *Parietaria judaica* (Pj) pollen has been isolated and sequenced from a cDNA library in lambda ZAP using a pool of 23 sera from Pj-allergic patients. The clone contained an insert of 622 nucleotides with an open reading frame of 133 amino acids (aa) and a putative signal peptide of 31 aa giving a deduced mature processed protein of 102 aa with a molecular mass of 11 344 Da. The expressed recombinant protein, named rPar j 2.0101, was a major allergen since it reacted with IgE of 82% (23/28) of the sera of Pj-allergic subjects analyzed. It was shown to be a new allergen since (i) the amino acid sequence homology with the already reported recombinant allergen Par j 1.0101 was 45% and (ii) there was no cross-inhibition between rPar j 2.0101 and rPar j 1.0101. In addition, rPar j 2.0101 inhibited 35% of the specific IgE for 10–14 kDa native allergens and preincubation of sera from Pj-allergic patients with both rPar j 2.0101 and rPar j 1.0101 fully abolished the IgE recognition of the 10–14 kDa native allergen region, suggesting that these two allergens contributed to the region.

Key words: cDNA cloning; Allergy; Immunoglobulin E; *Parietaria judaica*

1. Introduction

Parietaria judaica pollen is the main cause of allergy in the Mediterranean area and an epidemiological study carried out on allergic patients in Sicily showed that up to 50% reacted to Pj pollen extract [1,2]. The allergenic molecules contained in the pollen are usually a complex mixture of various proteins differing in either specific IgE binding or biochemical properties. The Pj pollen extract contains at least nine allergens having different molecular weights and IgE-binding specificity. The molecular mass ranges between 10 and 80 kDa [1–5] and the allergens found on the 10–14 kDa region reacted with the IgE of 95% of the sera from Pj-allergic patients tested, suggesting that major allergens are present in this region [6]. In order to plan a diagnostic and therapeutic approach to the allergic reaction, a preliminary step is to purify and to characterize each major allergen and a powerful tool towards this target is the molecular cloning of the allergens [7,8]. We have already cloned and characterized one of the major allergens of the *P. judaica* pollen that belongs to the 10–14 kDa region [9], Par j 1.0101, a protein of 139 amino acids and a predicted molecular mass of 14 509 Da. In this paper, we report the cloning, sequence, expression and allergenic activity of a new major allergen of Pj pollen named Par j 2.0101 according to the suggested allergen nomenclature [10].

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Abbreviations: Pj, *Parietaria judaica*; IgE, immunoglobulin E; RAST, radioallergosorbent test; cDNA, DNA complementary to mRNA; PCR, polymerase chain reaction.

EMBL data bank accession number P2:X95865.

2. Materials and methods

2.1. Total and poly(A)$^+$ RNA preparation

Total and poly(A)$^+$ RNA extracted from Pj flowers collected in Palermo, Italy, was prepared as described [9].

2.2. cDNA library screening and cDNA sequencing

Double-stranded cDNA was synthesized from mRNA by using oligo(dT) primers and cloned into the *Eco*RI and *Xho*I sites of the lambda ZAP expression vector, according to the Stratagene cDNA cloning kit (Stratagene, USA).

6×10^5 plaques were immunologically screened by using a pool of sera ($n = 23$) diluted 1:5 in PBS-containing 0.25% BSA, obtained from patients allergic to Pj pollen. Positive clones were identified by using ^{125}I-labelled anti-human IgE.

The nucleotide sequence of the cDNA insert was determined on both strands of the PBK-CMV plasmid vector by the dideoxy chain termination method using a Sequenase Kit (Amersham, USA).

2.3. Cloning in pMALc2

The oligonucleotides were synthesized by Pharmacia Biotech. Primers for PCR were (lower-case letters indicate the restriction enzyme cloning site; upper-case letters denote the coding sequence): (oligo 1) 5′ ccggaattcGAGGCTTGCGGGAAAGTGGTGCAGGAT 3′ (oligo 2) 5′ gtgtctagaATAGTAACCTCTGAAAATAGTACTTTGG 3′. 1 ng of the P2 clone was subjected to 30 cycles of PCR under the following conditions: 94°C 30 s, 52°C 30 s, 72°C 30 s.

The PCR product was fractionated on 1.8% agarose gel and after several steps of purification, digested with *Eco*RI and *Xba*I restriction enzymes. The fragments were cloned in the *Eco*RI/*Xba*I sites of the pMALc2 vector (Biolabs, UK). Recombinant clones were sequenced using the dideoxynucleotide chain termination method.

2.4. Preparation of the recombinant allergens

The recombinant clones were grown to 0.5–0.6 OD_{600} in LB broth and induced for 2 h with 0.3 mM isopropylthio-β-D-galactoside (IPTG). The cells were harvested by centrifugation (4000 rpm/20 min) and stored frozen. After thawing, the pellet was dissolved in sodium phosphate buffer (10 mM Na phosphate, pH 7.2; 200 mM NaCl, 1 mM EDTA, 1 mM NaN_3) and lysed by sonication using a Heat-System-Ultrasonic, Inc/W-385. The cell debris was then removed by centrifugation (9000 rpm/30 min) and the supernatant was diluted 1:20 with 10 mM EDTA and concentrated up to 2 mg/ml of total proteins by using a centriprep concentrator (Amicon) with a molecular mass cut-off of 10 kDa. The concentration of the recombinant allergens was detected by densitometry analysis of SDS-PAGE gels stained with Coomassie Brilliant Blue.

2.5. Western blot analysis

Protein samples were denatured under reducing conditions by boiling for 5 min in 50 mM Tris-HCl pH 6.8, 1% SDS, 2% β-mercap-

PII S0014-5793(96)01328-2

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                                                        M  R  T  V  S  M  A  A
P.j 2.0101  acagtctcagacacacccctttttccaaacttccaacATGAGGACCGTGTCGATGGCGGCA
            1                                                              60

             L  V  V  I  A  A  A  L  A  W  T  S  S  A  E  P  A  P  A  P
            CTCGTTGTGATCGCGGCGGCGCTCGCGTGGACATCTTCGGCTGAGCCGGCTCCAGCCCCG
                                                                       120

             A  P  G  E  E  A  C  G  K  V  V  Q  D  I  M  P  C  L  H  F
            GCCCCAGGAGAGGAGGCTTGCGGGAAAGTGGTGCAGGATATAATGCCGTGCCTGCATTTC
                                                                       180

             V  K  G  E  E  K  E  P  S  K  E  C  C  S  G  T  K  K  L  S
            GTGAAGGGGGAGGAGAAGGAGCCGTCGAAGGAGTGCTGCAGCGGCACGAAGAAGCTGAGC
                                                                       240

             E  E  V  K  T  T  E  Q  K  R  E  A  C  K  C  I  V  R  A  T
            GAGGAGGTGAAGACGACGGAGCAGAAGAGGGAGGCCTGCAAGTGCATAGTGCGCGCCACG
                                                                       300

             K  G  I  S  G  I  K  N  E  L  V  A  E  V  P  K  K  C  D  I
            AAGGGCATCTCCGGTATCAAAAATGAACTTGTCGCCGAGGTCCCCAAGAAGTGCGATATT
                                                                       360

             K  T  T  L  P  P  I  T  A  D  F  D  C  S  K  I  Q  S  T  I
            AAGACCACTCTCCCGCCCATCACCGCCGACTTCGACTGCTCCAAGATCCAAAGTACTATT
                                                                       420

             F  R  G  Y  Y  *
            TTCAGAGGTTACTATtagcaagttagagagcgttttctcttaatttctaagtgtggggaa
                                                                       480

            ctaaaaataaattatggcatgttacgttctataaggccatgttctttatatgaataatga
                                                                       540

            tgtattatgtgtgtaattgatattgatgtaataatgttaacaatacgatggaataatgtt
                                                                       600

            aacaatatgaaattgctactctaaaaaaaaaaaaaaaaa 3'
```

Fig. 1. Nucleotide and deduced amino acid sequence of the cDNA coding for the rPar j 2.0101 allergenic protein. Numbers in the right margin refer to the position of the nucleotides. The deduced amino acid sequence is shown using the single-letter code above the corresponding nucleotide triplet. The primers used for PCR are underlined.

(A)

```
                    1                                                  25
Par j 2.0101        M R T V S M A A L V V I A A A L A W T S S A E P A

                    26                                                 50
Par j 2.0101        P A P A P G E E A C G K V V Q D I M P C L H F V K
Par j 1.0101              Q - T - - T M - R A L - - - - P - - Q

                    51                                                 75
Par j 2.0101        G E E K E P S K E C C S G T K K L S E E V K T T E
Par j 1.0101        - K - - - - - - G - - - - A - R - D W - T - - G P

                    76                                                 100
Par j 2.0101        Q K R E A C K C I V R A T K G I S G I K N E L V A
Par j 1.0101        - R V H - - E - - Q T - M - T Y - D - D G K - - S

                    101                                                125
Par j 2.0101        E V P K K C D I K T T L P P I T A D F D C S K I Q
Par j 1.0101        - - - - H - G - V D S K L P P I D V N M D C - T L

                    126                                                150
Par j 2.0101        S T I F R G Y Y *
Par j 1.0101        G V V P - Q P Q L P V S L R H G P V T G P S D P A

Par j 1.0101        H K A R L E R P Q I R V P P P A P E K A *
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(B)

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                    34                                  50
Par j 1B            G P X G K V V X H I M P C L K F V K G
                      | | | |   | | | | | | | | | | | |
Par.j 2.0101        E A C G K V V Q D I M P C L H F V K G
                      |   | | | | | | | | |   | | | |
Par j 1A              A D G K V V Q D I M P P L L F V K
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Fig. 2. (A) Comparison between the rPar j 2.0101 and rPar j 1.0101 allergens, the putative signal peptide is underlined. (B) Comparison between the rPar j 2.0101 and two native isoforms of *Parietaria* allergens, i.e. Par 1A and Par 1B [5]. The dash indicates identity. Numbers refer to the amino acid sequence position of the Par j 2.0101 allergen.

toethanol, 0.01% bromophenol blue and separated by SDS-PAGE. The proteins were therefore electroblotted to Immobilon-P membranes (Millipore, USA) and then blocked for 3 h at room temperature in PBS supplemented with 3% BSA, 0.5% Tween and 0.02% NaN_3 and washed three times with PBS containing 0.1% (v/v) of Tween 20. Inhibition was carried out by adding to the diluted serum pool increasing amounts of recombinant proteins for 3 h at room temperature. The filters were then incubated overnight at room temperature with the serum pool previously absorbed. After a washing step, the filters were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgE (Sigma, St. Louis, MO). The final reaction was developed with an ECL detection system (Amersham, USA). The signal intensity was measured by using a Biorad densitometer (model GS-670).

3. Results and discussion

Ten clones capable of interacting with human IgE were isolated after screening of a lambda ZAP cDNA library using a pool of 23 sera of patients allergic to *P. judaica* pollen. Seven clones were still capable of binding human IgE after several steps of purification. The purified clones gave no IgE binding when tested with sera ($n = 5$) of subjects not allergic to Pj pollen. The sequence of the clones was then analyzed and one clone, named P2, was found to share an independent full-length cDNA. Sequence analysis of the P2 clone showed a 622 nucleotide insert with an open reading frame of 399 bp terminating with a TAG stop codon (Fig. 1). The cDNA contained a 36 nucleotide leader sequence and 184 nucleotide untranslated region with a canonical polyadenylation site and poly(A) tail. The cDNA insert encoded a protein of 133 aa with a deduced molecular mass of 14 105 Da. The hydrophobicity profile of the protein showed, in the amino-terminal region, a putative signal peptide of 31 aa specific for eukaryotic glycosylated proteins, giving a deduced mature processed protein of 102 aa with a molecular mass of 11 344 Da.

The recombinant allergenic protein named rPar j 2.0101 showed an homology of 45% with the aa sequence of the

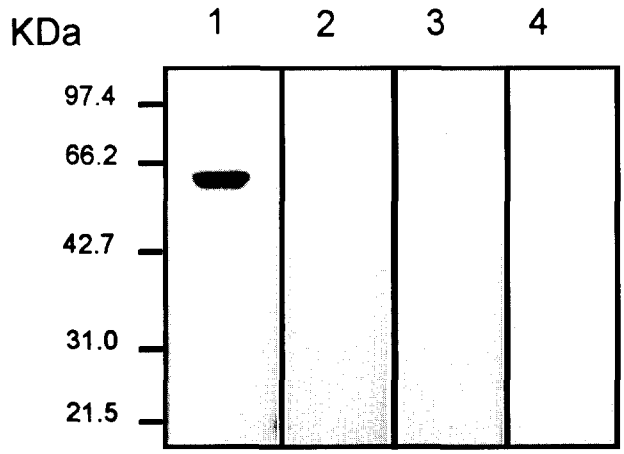

Fig. 3. Western blotting analysis. The recombinant allergen Par j 2.0101 (1 μg/lane) was run on 10% SDS-PAGE and immunoblotted as described in Section 2. Molecular mass markers (Biorad, USA) expressed in kDa are shown on the left. Lanes: 1, induced rPar j 2.0101 incubated with a pool of sera from Pj-allergic patients; 2, induced rPar j 2.0101 incubated with a pool of sera from non-allergic subjects; 3, non-induced rPar j 2.0101 incubated with a pool of sera from Pj-allergic patients; 4, induced pMAL-c2 incubated with a pool of sera from Pj-allergic patients. The pool of sera was diluted 1:5 with PBS-0.25% BSA. Specific IgE binding was detected with the HRP-conjugated anti-human IgE and ECL system.

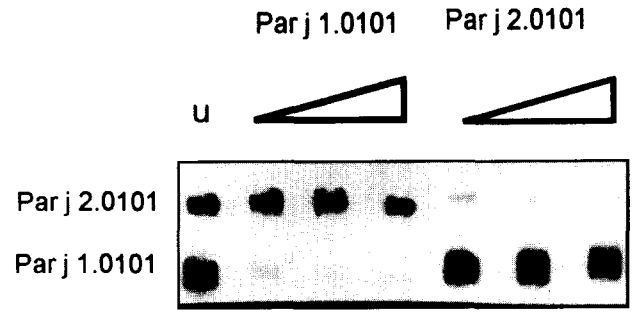

Fig. 4. Slot-blot inhibition. The recombinant allergens Par j 2.0101 and Par j 1.0101 were spotted (1 μg/slot) on nitrocellulose. The pool of sera from Pj-allergic patients diluted 1:5 was preincubated for 3 h at room temperature, with increasing amounts of recombinant allergen (1, 10, 50 μg/ml). U: pool of sera diluted 1:5 and preincubated with buffer alone as a control. Specific IgE binding was detected as reported in Fig. 3.

recombinant allergen Par j 1.0101 already reported [9] (Fig. 2A). In addition, from amino acids E32 to G51, rPar j 2.0101 showed significant homology (Fig. 2B) with the amino-terminal region of two native isoallergens, Par j 1A and Par j 1B, isolated by monoclonal antibody-based affinity chromatography and partially sequenced [5].

In order to achieve characterization at the molecular and immunological levels, the cDNA of the P2 clone after PCR was cloned without the putative signal peptide in the pMALc2 expression vector, giving a fused protein with a molecular mass of approx. 53 kDa. rPar j 2.0101 expressed as a protein fused to maltose binding protein (MBP), was capable of binding specifically the IgE from sera of patients allergic to *P. judaica* pollen, as shown by Western blot analysis (Fig. 3). The allergologic relevance of the recombinant Par j 2.0101 was determined by slot-blot analysis [11] using single sera ($n = 28$) from patients allergic to Pj pollen with a high level (RAST class 4+) of specific IgE. 82% (23/28 sera) of the sera showed IgE capable of binding the recombinant allergen Par j 2.0101, therefore, it can be classified as a major allergen (data not shown).

In order to assess the allergenic cross-reactivity between the recombinant allergens Par j 2.0101 and Par j 1.0101, the proteins were spotted on nitrocellulose membranes and incubated with a pool of 23 sera from Pj-allergic patients. Preincubation of the serum pool with increasing amounts of rPar j 2.0101 or rPar j 1.0101 completely abolished specific IgE binding to the same allergen without interference with the other and the results demonstrated in Fig. 4 strongly suggested that the two allergens showed a different IgE epitope composition.

The recombinant allergen Par j 2.0101 was capable of inhibiting approx. 35% of specific IgE binding to the 10–14 kDa native allergens group (Fig. 5). When preincubation of the serum pool was performed with both the recombinant Par j 2.0101 and Par j 1.0101, the binding of IgE to the 10–14 kDa native allergen region was totally inhibited, strongly suggesting that only those two allergens contributed to that region and that together they were capable of inhibiting the majority of the IgE specific for Pj allergens (Fig. 5).

Several isoforms of the major allergen Par j 1.0101 have been isolated and characterized (manuscript in preparation), while work is actually in progress in order to isolate isoforms for Par j 2.0101 as well.

Finally, a search made at the EMBL data bank showed that

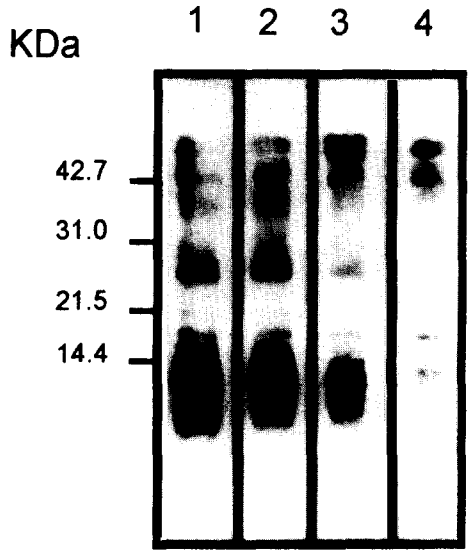

Fig. 5. Western blotting inhibition. The Pj pollen crude extract (5 μg/lane) was separated on 16% PAGE-SDS and immunoblotted as described in Section 2. Markers (Bio-Rad) expressed in kDa are shown on the left. Lanes: 1, serum pool preincubated with buffer alone as a control; 2, serum pool preincubated with 50 μg/ml of rPar j 2.0101; 3, serum pool preincubated with 50 μg/ml of rPar j 1.0101; 4, serum pool preincubated with 50 μg/ml of rPar j 2.0101+rPar j 1.0101. The inhibition procedure and IgE binding detection were performed as described in Figs. 3 and 4.

the recombinant allergen rPar j 2.0101 belongs to a family of protein referred to as non-specific lipid transfer protein (ns-LTPs) [12,13]. These proteins constitute a broad family capable of transferring lipid molecules through membranes.

In conclusion, in the present paper we have described the cloning and sequencing of a new allergen named Par j 2.0101. It is a major allergen since it was found to be capable of interacting with IgE of 82% of the Pj-allergic sera tested. Regarding the already cloned major Pj allergen rPar j 1.0101, rPar j 2.0101 is a different allergenic protein for the reason that it showed a different amino acid sequence and because there was no evident competition between the two recombinant allergens.

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