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cDNA cloning, expression and primary structure of *Par j* I, a major allergen of *Parietaria judaica* pollen

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Abstract

A 659 bp cDNA clone** coding for an allergen of Pj pollen has been isolated from a lambda gt11 library, and its DNA sequence determined. The cDNA insert showed an open reading frame of 429 bp coding for an allergenic protein of 14,866 Da and a deduced amino acid sequence containing 143 residues. The expressed recombinant protein represented the major allergen *Par j* I since it reacted with 95% of the sera from Pj-allergic patients ($n = 22$) and with two *Par j* I-specific monoclonal antibodies. No similarity with other known DNA and protein sequences has been detected.

Key words: cDNA; IgE antibody; Primary structure; Pollinosis; Recombinant allergen; *Parietaria judaica*

1. Introduction

Basic and clinical research as well as the diagnosis of allergy is usually hindered by the low amount of pure allergens available.

To overcome this limitation mRNA, isolated from different allergenic sources, is now used to generate cDNA libraries, and several recombinant allergens have been isolated [1–6]. However, to date a cloned *Parietaria* allergen has been not yet reported.

The pollen of the weed *Parietaria* is the main cause of allergic reactions in the Mediterranean countries [7–8], representing about 40% of the cases in pollen-allergic sensitive patients [9]. Its diffusion is not restricted to the Mediterranean area since *P. judaica* and *P. officinalis* have also been reported as a cause of allergic reactions in Eastern Australia [10], and in the United States respectively [11].

The allergenic composition of the *Parietaria* pollen has been studied [12–14] and the *P. judaica* aqueous extract is composed of a complex mixture of at least 26 antigens, 9 of which have been shown to bind specifically to IgE of allergic patients using either CRIE [15] or immunoblotting after transfer of SDS-PAGE separated proteins [16]. Among the 9 allergens, a major allergen named *Par j* I, has been purified to homogeneity by means of monoclonal antibody affinity chromatography and HPLC [17]. The purification of biologically active mRNA from Pj inflorescence and the sequence of the twelve N-terminal residues of the *P. officinalis* major allergen has been reported [18,19], while two *Par j* I isoforms have been recently separated and the partial N-terminal sequence reported. [20]. In this paper wedescribe for the first time the cloning, expression and sequencing of a cDNA encoding the major allergen *Par j* I, its deduced amino acid sequence and the allergenic activity.

2. Materials and methods

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

Inflorescence of Pj were collected from weeds flowering in Palermo Italy, and powdered after freezing in liquid nitrogen with a mortar and pestle.

The total RNA from the flowers was extracted as reported [21], with minor modifications. In short: the Pj-inflorescence powders were homogenised by vortexing with glass beads in lysis buffer (2% SDS, 1% 2-mercaptoethanol, 50 mM EDTA, 150 mM Tris-borate, pH 7.5), 3 ml buffer/g tissue and quickly mixed with 0.25 buffer volume of 100% ethanol and 0.9 buffer volume of 5 M potassium acetate (K^+). The mixing was performed for 1 min followed by chloroform extraction and centrifugation at 4°C for 30 min at 10,000 rpm.

The aqueous phase was extracted several times with phenol and finally with chloroform. The RNA was precipitated from the aqueous phase with 3 M LiCl containing 1% 2-mercaptoethanol (v/v), at −10°C overnight. The RNA was collected by centrifugation, and the pelletdissolved in 0.5 × lysis buffer plus ethanol/K^+ and extracted once in chloroform. Phenol/chloroform extractions were repeated several times until a clear interphase was obtained. One volume of 12M LiCl was added

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Abbreviations: Pj, Parietaria judaica; CRIE, crossed radio immunoelectrophoresis; IPTG, isopropylthio-β-D-galactoside.

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to the aqueous phase and left overnight at −10°C, then centrifuged and the pellet dissolved was again precipitated with 0.3 M sodium acetate, pH 5.5. After centrifugation, the pellet was resuspended in 1 ml H_2O. The solution was denatured at 65°C for 5 min in one volume of 2 × BB buffer (10 mM Tris pH 7.6, 0.5 M NaCl, 0.1% SDS), and chromatographed several times through a cellulose column previously sterilised with 0.3 N NaOH and equilibrated with 1 × BB buffer. The total RNA was eluted with 1 × BB buffer.

Poly(A)$^+$ mRNA was isolated by loading the total RNA solution onto an oligo(dT)-cellulose column (Sigma, St. Louis, MO), following the standard procedures reported [22].

2.2. cDNA library immunological screening and cDNA sequencing

Double strand cDNA was synthesised from mRNA using oligo dT primer and cloned into the *Eco*RI site of the phage vector lambda gt11, according to published procedures in the Amersham cDNA cloning kit (Amersham Corp., Arlington Heights, IL).

In brief, a cDNA library containing approximately 5×10^5 recombinant phages was constructed. 4×10^5 plaques were immunologically screened using pooled sera ($n = 10$) from patients allergic to Pj pollen and five positive IgE binding plaques were obtained. The sera from Pjallergic patients were tested with a radioallergosorbent test (RAST, Pharmacia, Sweden) and only sera with class four values were pooled and used in the experiments described in this paper. None of the five immunoreactive plaques showed any positive crossreactivity with sera from non-allergic subjects. Protein expression was induced and lysates prepared as described [23]. The nucleotide sequence of the cDNA insert was determined on both strands of pBluescript KS by the dideoxy sequencing procedure [24].

2.3. Western blot analysis

The lysates were separated by electrophoresis on a 10% SDS-PAGE gel, electroblotted to nitrocellulose filters and subsequently blocked for 3 h at room temperature in PBS, 3% BSA, 0.5% Tween and 0.02% NaN_3 and washed using PBS, 0.1% Tween solution.

The filters were immunoscreened using pooled sera from Pj-allergic and non-allergic subjects as a control and with two Par j I-specific monoclonal antibodies [17,25]. The monoclonals were diluted 1:5,000 in PBS, 0.25% BSA, 0.1% Tween and 0.02% NaN_3 and incubated at room temperature for 3.5 h, while the Pj-allergic and control non-allergic sera were diluted 1:5 in the same buffer. IgE- and IgG-allergen bound complexes were radio labelled using 5×10^5 cpm/ml rabbit anti-human IgE ^{125}I (Pharmacia, Sweden) and 17×10^3 cpm/ml sheep anti-mouse IgG ^{125}I (Amersham, UK), respectively, in PBS containing 0.25% BSA, 0.1% Tween and 0.2% NaN_3. The filters were extensively washed after every step in PBS and the radio labelled IgE-allergen or IgG-allergen complexes were detected by autoradiography.

2.4. Dot blot analysis

The same amount of the lambda gt11 P5 was spotted on a confluent Y1090 plate and left at 43°C until a small lysis plaque was visible (3 h). The wild type lambda gt11 was used as a negative control. A filter of nitrocellulose was then immersed in 10 mM IPTG, laid on the plate and transferred at 37°C for 4 h to induce the recombinant P5-β-galactosidase protein. The filters were blocked and treated as above described.

2.5. Northern blot analysis

The total cellular and poly(A)$^+$ RNAs have been prepared as described above [21,22]. RNAs were fractionated on a 1% agarose-formaldehyde gel and transferred to Hybond-N filters as recommended by manufactures. The hybridisation was carried out at 65°C as reported [26] and the filters were washed under high stringency conditions.

3. Results and discussion

The total RNA was isolated from Pj-flowers with a yield of about 0.5 mg per gram of wet flowers. The poly(A)$^+$ mRNA purified by affinity chromatography was used as template to synthesise the complementary

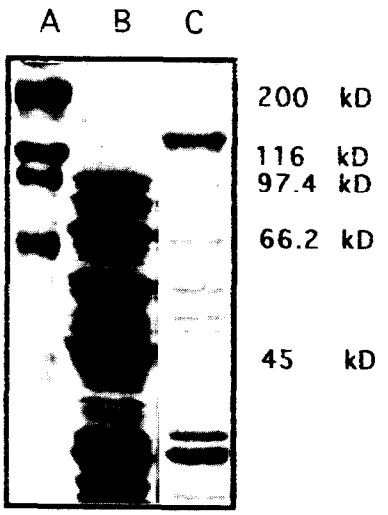

Fig. 1. Comassie blue stained gel of total cell lysates of P5 lambda gt11 clone. Lane A: molecular weight markers; lane B: non-induced recombinant lysates; lane C: induced recombinant lysates.

DNA, 20 ng of cDNA were used for constructing a Pj-specific cDNA library in the expression vector lambda gt11. About 4×10^5 plaque-forming units of recombinant phages were immunoscreened for IgE-reactive plaques by using sera ($n = 10$) from patients allergic to Pj pollen and five putative positive clones were found during the first screening. The screening with non-allergic sera ($n = 10$) used as a control, gave no positive reaction. One of five positive clones, designated as P5, was further used to induce a recombinant fusion protein.

From the recombinant P5 clone, an approximately 129 kDa P5-β-galactosidase fusion protein can be visualised in the total cell lysate after IPTG induction (Fig. 1, lane C). The 129 kDa recombinant protein was composed of a 114 kDa fragment of β-galactosidase fused to the P5 protein. Lane B shows a total cell lysates from recombinant P5 clone without induction, while on Lane A a pool of protein markers (range 45–200 kDa) was used.

Fig. 2 panel A represents a Western blot of the same cell lysates from the recombinant P5 clone showed in Fig. 1. Lanes 1 and 2 show immunoblot screened with pooled allergic sera with non-induced and induced P5 protein, respectively. Only the induced sample was capable of binding with the Pj-allergic sera. Lane 3 represents a pool of protein markers including the β-galactosidase protein as a control and used at the same concentration as in Fig. 1. No IgE-binding activity was detected for this protein confirming that the P5-cDNA clone itself encoded for an IgE-reactive allergen. Lanes 4 and 5 are immunoblots of non-induced and induced P5 clones screened with a pool of non-allergic sera. No IgE complexes were found supporting the specificity of the immuno reaction reported above. A high number of antigens ($n = 26$) of the Pj pollen has been already partially characterised [15–16].

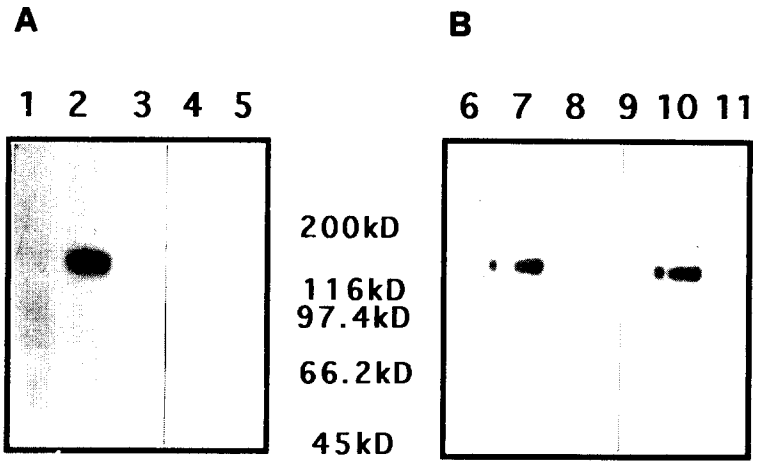

Fig. 2. SDS-PAGE gel of total cell lysates from recombinant *E. Coli.* (Panel A) lane 1: non-induced recombinant lysates; lane 2: induced recombinant lysates; lane 3: molecular weight markers; Lanes 1, 2 and 3 were immunoblotted using a pool of sera ($n = 22$) from Pj-allergic patients; lane 4: non-induced recombinant lysates; lane 5: induced recombinant lysates; lanes 4 and 5 were immunoblotted using a pool of sera ($n = 10$) from non-allergic subjects. (Panel B) lane 6: non-induced recombinant lysates; lane 7: induced recombinant lysates; Lane 8: molecular weight markers; lane 9: non-induced recombinant lysates; lane 10: induced recombinant lysates; lane 11: molecular weight markers. Panel B lanes were immunoblotted using two different monoclonal antibodies specific for the *Par j* I major allergen (see section 2 for details).

At least 9 of them were capable of binding specific human IgE and 4 were classified as major allergens [15–16]. To evaluate which of them we had isolated, we performed an immunoblot screened with two monoclonal antibodies specific for the *Par j* I allergen [17,25] and the result is reported in Fig. 2 panel B. Lanes 6 and 9 show non- induced total cell lysates, lanes 7 and 10 the induced total cell lysates, while lanes 8 and 11 the molecular weight markers. The very specific immunoreaction between the induced P5 protein and the two monoclonal antibodies showed in lanes 7 and 10, demonstrated that the P5- cDNA clone encoded for the major allergen *Par j* I. In addition, to study the clinical relevance and diffusion of the P5 expressed protein, 22 sera from patients not in therapy and allergic to the Pj pollen, were individually tested in a dot blot assay (see section 2 for details). Of all the sera, 95% showed a strong IgE-binding interaction suggesting that the P5 expressed protein clearly represents a major allergen. The negative IgE-interaction observed for the remaining 5% of the sera could be due to the possibility that they did not share any IgE specific for the *Par j* I, or that the recombinant technology made the P5 protein unreactive versus some specific allergic sera. These results are in agreement with those obtained using the native *Par j* I capable of reacting with 95–100% of the Pj-allergic sera [15–16]. The cDNA insert of the P5 clone was then excised from the lambda gt11 phage after *Eco*RI digestion and subcloned into pBluescript KS (Stratagene). The DNA sequence analysis revealed a 659 bp cDNA clone (Fig. 3) with an open reading frame of 429 nucleotides and with a deduced amino acid sequence of 143 residues (starting from the first methionine). The P5 clone also showed a 185 bp 3′ untraslated region with a canonical poly(A)$^+$ tail at the end. The molecular weight of the P5 protein is of 14,866 kDa which corresponds to the previously detected molecular weight of the native *Par j* I allergen [16]. To confirm that the P5 clone represented a full length clone, a Northern blot from total and poly(A)$^+$ RNAs from Pj flowers has been performed. Fig. 4 shows the hybridisation pattern and the native size of the P5 clone mRNA (lanes A and B). The band showed a size of about 650 bp as expected. The RNAs used in lanes A and B were nucleic acids from flowers collected in May, while lanes C and D collected in January. Lanes A,B and C,D showed a different level of expression suggesting that the P5 cDNA clone has a pattern of expression as expected for a seasonal flowering plant.

In conclusion in this paper we describe the isolation and characterisation of the cDNA clone coding for the major allergen *Par j* I. A recent paper published by Ayuso et al. [20] reports the NH_2-terminal sequence of two *Par j* I isoforms (*Par j* I A: 17 a.a.; *Par j*I B: 19 a.a.). The comparison of the two short reported sequences with the P5 protein showed a region of high homology starting from the 10th amino acid residue of the isoform A. The differences observed in the remaining region could be due to the different isoforms analysed by the two groups or to intraspecie variation.

The data presented in this paper might represent a useful step towards further basic and clinical research on the *Parietaria*-induced allergy. The primary structure of the *Par j*I, one of the major allergenic molecule of the Pj pollen, would allow the preparation of synthetic peptides useful for either B and T epitope detection. Cell culture and animal studies suggest that peptides representing epitopes on allergen can be used to induce tolerance to allergens. Administration of these substances to patients

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                                                    M  V  R  A  L  M  P  C  L  P  F  V
gaattcCAAGAAACCTGCGGGACTATGGTGAGAGCGCTGATGCCGTGCCTGCCGTTCGTG
                                                                                  60

Q  G  K  E  K  E  P  S  K  G  C  C  S  G  A  K  R  L  D  G
CAGGGGAAAGAGAAAGAGCCGTCAAAGGGGTGCTGCAGCGGCGCCAAAAGATTGGACGGG
                                                                                 120

E  T  K  T  G  P  Q  R  V  H  A  C  E  C  I  Q  T  A  M  K
GAGACGAAGACGGGGCCGCAGAGGGTGCACGCTTGTGAGTGCATCCAGACCGCCATGAAG
                                                                                 180

T  Y  S  D  I  D  G  K  L  V  S  E  V  P  K  H  C  G  I  V
ACTTATTCCGACATCGACGGGAAACTCGTCAGCGAGGTCCCCAAGCACTGCGGCATCGTT
                                                                                 240

D  S  K  L  P  P  I  D  V  N  M  D  C  K  T  L  G  V  V  P
GACAGCAAGCTCCCGCCCATTGACGTCAACATGGACTGCAAGACAGTTGGAGTGGTTCCT
                                                                                 300

R  Q  P  Q  L  P  V  S  L  R  H  G  P  V  T  G  P  S  R  S
CGGCAACCCCAACTTCCAGTCTCTCTCCGTCATGGTCCCGTCACGGGCCCAAGTCGATCC
                                                                                 360

R  P  P  T  K  H  G  W  R  D  P  R  L  E  F  R  P  P  H  R
CGCCCGCCCACAAAGCACGGTTGGAGAGACCCCAGATTAGAGTTCCGCCCCCCGCACCGG
                                                                                 420

K  K  P  N  P  A  F  S  T  L  G
AAAAAGCCTAACCCAGCTTTCTCCACTCTTGGATGAGCGAGGCTAATATTATTAAGCCCT
                                                                                 480

AAACTCTAATGACGGTGGAACTGTGTTATGAGAAGTTATATGCAATAATTATCTTAATAT
                                                                                 540

ATATATATATATATATAGTTGACTAGTGAGTAGTGACATCATTCACGTAATAAATAATGT
                                                                                 600

GTACTTTACTTATCGATATAATGATTAAAGATATGTTGATTAAAAAAAAAAAgaattc
                                                                                 659
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Fig. 3 Nucleotide and deduced amino acid sequences of the P5 cDNA coding for the *Par j* I allergenic protein. Numbers in the right margin refer to positions of the nucleotides. The deduced amino acid sequence is shown by one-letter code above the corresponding codon. Lower case letters show the *Eco*RI cloning site. The underlined trinucleotide shows the termination codon.

can down regulate T cells and the consequent inflammatory response and greatly reduce the risk of anaphylaxis that the standard immunotherapy may induce. The recombinant allergen could also be a good tool for further immunochemical studies and for the diagnosis of the most widespread Mediterranean allergy to pollen.

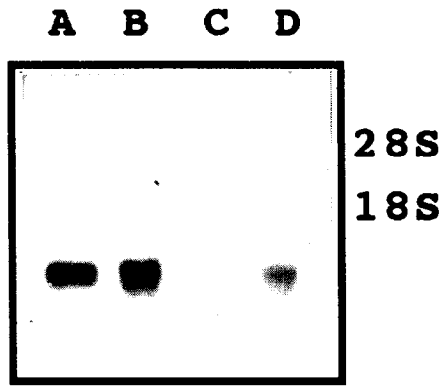

Fig. 4 Northern blot analysis of *Parietaria judaica* RNA flowers. Lane A: 0.5 μg of poly(A)$^+$RNA; lane B: 20 μg of total RNA; lane C: 0.5 μg of poly(A)$^+$RNA; lane D: 20 μg of total RNA. Lanes A and B show RNAs from flowers collected in May, Lanes C and D in January.

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