Available online at www.sciencedirect.com
ScienceDirect
Molecular Immunology 45 (2008) 2465–2473
Molecular Immunology
www.elsevier.com/locate/molimm

Isolation, expression and immunological characterization of a calcium-binding protein from *Parietaria* pollen

A. Bonura [a], L. Gulino [a], A. Trapani [a], G. Di Felice [b], R. Tinghino [b], S. Amoroso [e], D. Geraci [a], R. Valenta [c], K. Westritschnig [c], E. Scala [d], A. Mari [d], P. Colombo [a,*]

[a] *Istituto di Biomedicina ed Immunologia Molecolare "Alberto Monroy" del Consiglio Nazionale delle Ricerche, Via Ugo La Malfa 153, Palermo, Italy*
[b] *Dipartimento di Malattie Infettive, Parassitarie e Immuno-mediate, Istituto Superiore di Sanità, Roma, Italy*
[c] *Christian Doppler Laboratory for Allergy Research, Division of Immunopathology, Department of Pathophysiology, Medical University of Vienna, Austria*
[d] *Center for Clinical and Experimental Allergology, IDI-IRCCS, Roma, Italy*
[e] *Unità Operativa di Allergologia, Ospedale Civico, Palermo, Italy*

Received 26 October 2007; received in revised form 10 January 2008; accepted 11 January 2008
Available online 4 March 2008

Abstract

The diagnosis and therapy of allergic disorders are usually performed with crude extracts which are a heterogeneous mixture of proteins with different allergenic potency. The knowledge of the allergenic composition is a key step for diagnostic and therapeutic options. *Parietaria judaica* pollen represents one of the main sources of allergens in the Mediterranean area and its major allergens have already been identified (Par j 1 and Par j 2). In addition, inhibition studies performed using a calcium-binding protein (CBP) from grass pollen (Phl p 7) showed the presence of a homologue of this cross-reactive allergen in the *Parietaria* extract. Screening of a cDNA library allowed us to isolate a 480 bp cDNA containing the information for an 87 AA long protein with high level of homology to calcium-binding proteins from other allergenic sources. It was expressed as a recombinant allergen in *Escherichia coli* and purified by affinity chromatography. Its expression allowed us to study the prevalence of this allergen in a population of allergic patients in southern Europe. Immunoblotting and inhibition studies showed that this allergen shares a pattern of IgE epitopes in common with other 2-EF-hand calcium-binding proteins from botanically non-related species. The immunological properties of the Pj CBP were investigated by CD63 activation assay and CFDA-SE staining. In conclusion, DNA recombinant technology allowed the isolation, expression and immunological characterization of a cross-reactive calcium-binding protein allergen from *Parietaria judaica* pollen.

Keywords: Cross-reactivity; EF-hand; *Parietaria* pollen; Polcalcin; Recombinant allergen

1. Introduction

The diagnosis of Type I allergic reaction is based on both the clinical history and presence of IgE in the serum and/or on skin mast cells. The use of extract-based tests for diagnosis represents a powerful tool but it is not sufficient to identify the molecules inducing an allergic response and, in some cases, it is not useful to distinguish between co-recognition to different allergenic molecules and cross-reactivity between allergens. On the other hand, diagnostic tests performed with purified natural and/or recombinant allergens can allow the establishment of a patient's molecular pattern of IgE recognition (Hiller et al., 2002). Therefore, the knowledge of the complete spectrum of allergens contained in an allergenic source represents a key issue for diagnosis and formulation of immunotherapy vaccines (Mothes et al., 2006; Valenta and Niederberger, 2007).

Pan-allergens (i.e. calcium-binding proteins (CBPs), profilins and lipid transfer proteins) are proteins with highly conserved amino acid sequences widely distributed even in botanically unrelated sources, therefore accuracy of diagnostic tests can be difficult due to their allergenic cross-reactivity (Vieths et al., 2002).

In particular, distinct proteins containing calcium-binding domains have been identified as a family of highly cross-

* Corresponding author. Tel.: +39 0916809535; fax: +39 0916809548.
E-mail address: paolo.colombo@ibim.cnr.it (P. Colombo).

0161-5890/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2008.01.009

reactive allergens in pollens of the most common allergenic plants (grasses, trees, and weeds) (Wopfner et al., 2007). Many CBPs belong to the same evolutionary family and share a type of calcium-binding domain known as the EF-hand (Kawasaki et al., 1998). This type of domain consists of a 12 residues loop flanked on both sides by a 12 residues α-helical domain. The basic structural/functional unit of EF-hand proteins is usually a pair of EF-hand motifs that form a stable four-helix bundle domain. Despite the large number of CBPs identified so far, only a few of them display allergenic activity (Wopfner et al., 2007). According to the number of EF hands contained in their sequences, they can be classified into three groups. Two EF-hand allergens were described in pollens from birch (Bet v 4) (Engel et al., 1997; Twardosz et al., 1997) alder (Aln g4) (Hayek et al., 1998), olive (Ole e 3) (Batanero et al., 1996), bermuda grass (Cyn d 7) (Smith et al., 1997; Suphioglu et al., 1997) and timothy grass (Phl p 7) (Niederberger et al., 1999). A pollen-specific allergen with 3-EF-hands, Bet v 3, was cloned from birch pollen (Seiberler et al., 1994), and allergens with 4-EF-hands were isolated from prickly juniper (Jun o 4) (Tinghino et al., 2002) and olive pollen (Ole e 8) (Ledesma et al., 2000). So far, the Allergome database (www.allergome.com, accessed on 30 August 2007) lists 51 polcalcin entries with allergenic activity, most of them identified by immunochemical methods. In particular, the two EF-hand allergens represent proteins with a molecular mass of 8–9 kDa, which rapidly elute from pollen grains and induce severe allergic reactions in sensitized patients. CBPs have been reported as capable of inducing a strong skin test reactivity if present in pollen allergenic extracts, and to be more frequently associated with severe asthma symptoms compared to profilins (Mari, 2001). It has been shown that the two EF-hand allergen from timothy grass pollen, Phl p 7, contains the majority of IgE epitopes of pollen-derived calcium-binding allergens (Tinghino et al., 2002), and its three-dimensional structure has been solved (Verdino et al., 2002).

Parietaria allergy is one of the most relevant type of pollen allergy in the population living in the Mediterranean basin (D'Amato et al., 2007). About 30% of all the allergic subjects in southern Italy present a skin prick test (SPT) reactivity to the *Parietaria judaica* (Pj) pollen extract (D'Amato, 2000). The composition of the allergenic extracts of the Pj pollen has been studied by biochemical, immunochemical and molecular biology methods and the two major allergens (Par j 1 and Par j 2) have been isolated and characterized (Colombo et al., 2003). Par j 1 and Par j 2 allergens are two small proteins belonging to the non-specific lipid transfer protein (nsLTP) family (Colombo et al., 1998). Immunoblot experiments and quantitative IgE measurement demonstrated that these two proteins are the major elicitors of IgE response in *Parietaria* allergic subjects (Costa et al., 1994; Duro et al., 1996; Stumvoll et al., 2003) and are the marker allergens capable of predicting a genuine sensitization to the Pj pollen (Stumvoll et al., 2003). Furthermore, inhibition studies performed using two recombinant allergens from grass (Phl p 7) and birch (Bet v 2) pollens have shown the presence of two homologues of these two highly cross-reactive allergens in the Pj extract (Stumvoll et al., 2003). By molecular cloning, two isoforms of *Parietaria* profilin have recently been isolated (Asturias et al., 2004).

In this report we describe the isolation, expression in *Escherichia coli* and immunological characterization of a 2-EF-hand polcalcin from *Parietaria judaica* pollen.

2. Experimental

2.1. Screening of the cDNA library

A *Parietaria judaica* cDNA library (Lambda ZAP, Stratagene, USA) was prepared as previously described (Duro et al., 1997). The library was screened with a Phl p 7 cDNA clone (Niederberger et al., 1999) labelled with $dCTP^{32}$. P^{32} labelled phages were picked, plaque-purified, and stored at 4 °C. Inserts from the selected phage were amplified with M13 forward and M13 reverse primers by PCR (94 °C 1 min, 52 °C 1 min, 72 °C 1 min for 30 cycles), cloned in the pCR4-TOPO vector (Invitrogen, USA) and sequenced. The full-length sequence of the cDNA clones was obtained by using the GeneRacer kit (Invitrogen, USA). The kit ensures the amplification of only full-length transcript via elimination of truncated messages from the amplification process. 5′ RACE was performed by PCR (94 °C 1 min, 52 °C 1 min, 72 °C 1 min for 30 cycles) using a PjCBP-specific oligonucleotide 5′ATCTCGGCCATCATGCG3′ (see Fig. 1 for details) and the GeneRacer™ 5′ nested oligonucleotide 5′GGACACTGACATGGACTGAAAGGAGTA3′. Fragments were purified, ligated in the pCR4-TOPO vector (Invitrogen, USA) and sequenced. Similarity searches and alignments of deduced amino acid sequences were performed using the BLAST 2.0 program (www.ncbi.nlm.nih.gov/BLAST/). Similarity structures were obtained by using the services of the Swiss-Model Protein Modelling server (http://swissmodel.expasy.org) using as a template the structure of the Phl p 7 allergen (entry 1K9UA).

2.2. Expression and purification of the recombinant Pj CBP protein

Recombinant Pj CBP was expressed as His-tagged fusion protein using the pQE30 expression vector (Qiagen, UK). In particular, the *Parietaria* CBP coding region was amplified using the following primers: CBP forward 5′cgcGGATCC**ATG**-GCCGACAAGCAGA3′ and CBP reverse 5′cgcTTCGAA**TCA**-GAAGATCTTGGC3′ (underlined letters indicate the BamH1 and HindIII restriction enzyme sites introduced for cloning in the expression vector, bold letters the first methionine and the stop codon, respectively) (see Fig. 1 for details).

DNA fragments were purified, digested with the appropriate restriction enzymes and cloned in the BamHI and HindIII restriction sites of the pQE30 vector (Qiagen, UK). Competent *E. coli* M15 cells (Qiagen, UK) were transformed and the coding reading frame of the recombinant clones checked by sequencing. The clones were grown over night at 37 °C in 2YT broth (Bacto-tryptone 16 g/l, Bacto-yeast 10 g/l, NaCl 5 g/l, pH 7.0). A 1:40 dilution was made and the culture was grown for 2 h at 37 °C and, after that, induced with isopropyl-D-thiogalactopyranoside