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A mutant of the major melon allergen, Cuc m 2, with reduced IgE binding capacity is a good candidate for specific immunotherapy

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ABSTRACT

Hypoallergenic mutants with reduced IgE-binding capacity but which show a similar T-cell response to the corresponding natural allergen are ideal tools for immunotherapy, for preventing a possible anaphylactic shock. An IgE conformational epitope has been identified in Cuc m 2, the major allergen and profilin from melon. Since this epitope is highly conserved in most pollen profilins, it may contribute to an explanation of cross-reactivity between pollen and food profilins. Mutants (Mut 1 and Mut 2) were generated by changing specific residues of the Cuc m 2 epitope to alanine, produced in *Escherichia coli*, and purified by chromatographic methods. Mut 1 showed a slight reduction in IgE binding but an allergenic activity that was similar to recombinant Cuc m 2, as measured by basophil activation test (BAT) and skin prick test (SPT). By contrast, Mut 2 displayed a substantial reduction in IgE-binding capacity (57%) and positive responses, as determined by BAT (33%) and SPT (50%), when compared to those of rCuc m 2. However, the T-cell proliferation and cytokine production induced by Mut 2 and rCuc m 2 were similar. Thus, this mutant represent potential candidate for immunotherapy of profilin allergies.

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1. Introduction

More than one-third of people living in industrialized nations suffer from some kind of allergy. The cause of the indisputable increase in the prevalence of allergies is still unknown. Allergies are considered to be not only a major health problem due to their negative impact on quality of life, but also constitute an economic burden in western countries (Wills-Karp et al., 2001). The greatest increase in this kind of illness has been observed among children, a trend which makes it all the more important to find effective treatments. Specific immunotherapy is the only currently approved curative treatment available, although it has been associated with side effects, especially those arising from the use of

raw extracts. However, the availability of purified allergens and the endless supply of recombinant forms could solve this problem. Nevertheless, wild-type molecules still have the capacity to cross-link pre-existing IgEs, thereby triggering anaphylactic reactions. For this reason, approaches to the production of modified allergens with reduced IgE-binding capacity (hypoallergens) provide a solution to problems associated with immunotherapy (Crameri, 2007; Ferreira et al., 2004; Singh and Bhalla, 2003; Valenta, 2002; Westritschnig et al., 2007). These modified hypoallergens preserve the sequence and structural motifs necessary for T cell recognition and for inducing IgG antibodies that react with the natural allergen. Two mechanisms have been described for generating mutants, one in which the mutations retain the structure of the protein and another in which the IgE epitopes are altered through protein folding. Although protein folding may be modified by mutations, both strategies are valid for use in immunotherapy.

Profilins are small cytosolic proteins (12–15 kDa) that are associated with cytoskeletal reorganization due to their ability to bind actin and phosphatidyl inositol-4,5-bisphosphate (Schluter et al., 1997; Witke, 2004), since these are involved in processes that are essential for cell survival. Members of this protein family are present in most organisms that have been investigated and

Abbreviations: BAT, basophil activation test; PBMCs, peripheral blood mononuclear cells; SI, stimulation index; SPT, skin prick test; TCLs, T cell lines; 3-D, three dimensional.

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Table 1

Demographic data, basophil activation test and skin prick test to rCuc m 2, its mutants and Pho d 2 in patients with melon allergy.

Patient	Age years	Sex	Pollen allergy	Melon allergy	Basophil activation test 0.01 µg/mL (SI) ^a				Skin prick test ^b		
					Cuc m 2	Mut 1	Mut 2	Pho d 2	Cuc m 2	Mut 1	Mut 2
1	18	F	RC,A	OAS	nd	nd	nd	nd	6 × 8	4 × 5	–
2	24	F	RC,A	OAS	3.3	1.9	1.3	3.2	5 × 5	5 × 6	4 × 5
3	24	F	RC	OAS	18.7	2.7	2.0	43.0	5 × 3	4 × 4	–
4	25	F	RC	OAS	11.8	5.8	1.3	25.0	4 × 3	3 × 3	–
5	19	M	RC	OAS	4.2	5.0	0.7	6.0	8 × 5	5 × 6	6 × 3
6	28	F	RC,A	OAS	nd	nd	nd	nd	10 × 6	4 × 4	–
7	25	M	RC	OAS	30.3	26.8	31.9	31.6	nd	nd	nd
8	18	M	RC	OAS	9.0	17.8	4.8	6.7	12 × 10	20 × 10	9 × 5
9	24	M	RC,A	OAS	nd	nd	nd	nd	5 × 6	6 × 8	4 × 3
10	41	M	RC	OAS	nd	nd	nd	nd	nd	nd	nd
11	37	M	RC, A	OAS	nd	nd	nd	nd	nd	nd	nd
12	33	F	RC, A	OAS	nd	nd	nd	nd	nd	nd	nd
13	21	M	RC	OAS	nd	nd	nd	nd	nd	nd	nd

A, asthma; OAS, oral allergy syndrome; RC, rhinoconjunctivitis; F, female; M, male; SI, stimulation index; nd, not determined or not included (Cuc m 2 < 2.5); –, negative response.

^a SI > 2.5 was considered a positive response.

^b SPT > 3 mm × 3 mm was considered positive.

constitute a family of highly conserved panallergens (i.e., amino acid sequence identity greater than 70% in plant members) (Lopez-Torrejon et al., 2005; Radauer, 2004). Recognized by 10–30% of patients allergic to pollen and/or plant foods, they are usually considered minor allergens in Central and North Europe. However, in South Europe, they are considered to be major allergens, particularly in some plant foods such as melon, which is responsible for one of the most common allergies (Cuesta-Herranz et al., 2000), and they are frequently associated with sensitization to different pollens, especially those of grasses (Barber et al., 2008; Cuesta-Herranz et al., 2010; Pastor et al., 2009). Cross-reactivity between profilins may explain the high correlation between these allergies. So, patients sensitized to these allergens have problems with a wide panel of foods and pollens. This fact supposes a very important loss of quality of life. Specific immunotherapy based on profilins could lead to the better treatment of this type of patient.

Cuc m 2, the melon profilin, is recognized by 80% of allergic patients (Lopez-Torrejon et al., 2005; Santos and Van Ree, 2011) in Spain. Lopez-Torrejon et al. (2005) produced this allergen as a recombinant protein in the *Escherichia coli* system, thus demonstrating the equivalence of the natural and recombinant proteins. Both purified forms of melon profilin were recognized by rabbit anti-profilin antibodies and IgE of sera from allergic patients, and also showed molecular sizes that are typical of the profilin family. The characterization of Cuc m 2 IgE epitopes has been addressed by means of two complementary strategies (Lopez-Torrejon et al., 2007; Tordesillas et al., 2010). Using overlapping synthetic peptides bound to a membrane, four major epitopes were identified: two with stronger IgE-binding capacity (E1, 66–75 and 81–93, and E2, 95–99 and 122–131) partially overlapped with the actin-binding site; and two weak epitopes (E3, 2–10, and E4, 35–45) (Lopez-Torrejon et al., 2007). In addition, an IgE conformational epitope was defined (Tordesillas et al., 2010) which was highly conserved in birch Bet v 2 and timothy grass Phl p 12 profilins (12/14 residues). This identity could explain the prevalent cross-reactivity detected among the members of this family. This epitope was not conserved in less closely related members, such as human profilin PLP1 (4/14 residues).

Based on these studies, we designed two mutants of Cuc m 2, using site-directed mutagenesis. Both new mutants had different IgE binding capacities, Mut 2 having the lowest allergenic activity. However, both retained their capacity to stimulate T cells and had similar cytokine profiles. Their potential use for immunotherapy of grass-melon allergy is discussed.

2. Methods

2.1. Patients and sera

Sera from 13 patients with melon allergy and from six tolerant controls were collected for this study at the Servicio de Alergia, Hospital de Basurto (Bilbao, Spain; Table 1, patients 1–9) and at the Servicio de Alergia, IIS-Fundación Jiménez Díaz (Madrid, Spain; Table 1, patients 10–13). All patients suffered oral allergy syndrome without systemic reactions and showed a positive response to rCuc m 2 with SPT and ELISA. Six non-melon-allergic control volunteers confirmed their non-IgE status with respect to recombinant Cuc m 2, as determined by ELISA. The study was approved by the Ethics Committees of Hospital de Basurto and IIS-Fundación Jiménez Díaz and written informed consent was obtained from patients and controls.

2.2. Design of the Cuc m 2 mutants

Site-directed mutagenesis to generate Cuc m 2 mutants was performed with appropriate PCR amplification primers and, as a template, the previously obtained cDNA encoding Cuc m 2 (Lopez-Torrejon et al., 2005). All substitutions were made to alanine with the exception of the A₅ residue, which was replaced with glycine.

The PCR products coding the mutant cDNAs were inserted into the pRSET plasmid (Invitrogen, Paisley, UK) to transform *E. coli* XL1-Blue cells, as previously described (Lopez-Torrejon et al., 2005). Several clones of each mutant were sequenced using the ABI PRISM Dye Terminator Kit and an ABI 3100 sequencer (Perkin-Elmer Biosystems, Warrington, UK).

2.3. 3D folding analysis of native and mutant form of Cuc m 2

Mut 2 was chosen to analyze 3D folding because this mutant has more changed residues than Mut 1. The initial geometry for Cuc m 2 protein was the previously reported structure (Lopez-Torrejon et al., 2007). The starting geometry for the mutated protein was obtained changing amino acids 2, 9, 10, 111–114, 116, 117, 121 to alanine with the Mutate tool of Swiss-PDB Viewer 4.01 program (Guex and Peitsch, 1997). Both geometries were then optimized using NAMD calculations (Phillips et al., 2005) with VMD 1.8 (Humphrey et al., 1996). The optimizations were terminated when total energies were stabilized, which happened after 15,200 steps

in Cuc m 2 and 19,900 steps in Mut 2. Both optimized structures were superposed with PyMOL 1.4 (LLC).

2.4. Production of native and mutant form of Cuc m 2

Wild type was produced following previously described methods (Lopez-Torrejon et al., 2005) and purified from *E. coli* BL21 lysed cells by affinity chromatography on a poly-L-proline-Sepharose 4B column. The mutants were obtained from the soluble fraction of induced cells separated by gel filtration on a Superdex HR 75 16/26 column (Pharmacia Biotech) by using 100 mM ammonium acetate (pH 6.8) at 1 ml/min. Peaks containing wild type or mutant forms were identified by SDS–PAGE and immunodetection with polyclonal anti-profilin antibodies (see below).

Protein purity was measured by SDS–PAGE, N-terminal amino acid sequencing with an Applied Biosystems 477A gas-phase sequencer (Applied Biosystems, Foster City, CA, USA), mass spectrophotometric analysis with a Biflex III Spectrometer (Bruker-Franzen Analytik, Bremen, Germany), and by fingerprinting after tryptic digestion, using standard methods. The concentration of proteins was calibrated by amino acid analysis following the standard ion-exchange chromatographic method in a Pharmacia Biochrom 20 analyzer.

2.5. SDS–PAGE and immunodetection assays

Samples (3 µg of wild type, Mut 1, and Mut 2) were separated by SDS–PAGE (Laemmli, 1970) and replica gels were electrotransferred onto polyvinylidene difluoride (PVDF) membranes. After blocking, the membranes were incubated overnight with a pool of sera from patients (1:2 dilution; Table 1) or with rabbit polyclonal antibodies to watermelon profilin (1:10,000 dilution; 1 h; antibodies kindly provided by Dr. C. Pastor, IIS-Fundación Jiménez Díaz, Madrid, Spain). Detection of IgE/IgG-binding components was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Amersham Biosciences, Little Chalfont, UK).

2.6. Specific IgE binding and ELISA inhibition assay

Specific IgE to wild type and mutants (range, 10–0.15 µg/ml) was determined using individual sera from seven melon-allergic patients (Table 1, patients 7–13; 1:2 dilution), a pool of sera from the same patients and a pool obtained from control subjects (1:2 dilution) by ELISA (Diaz-Perales et al., 2000). Blocking solution (Sigma, Steinheim, Germany) without solid phase extraction was used as a negative control, and optical density (OD) values > 0.1 units ($n = 6$; mean + 3 × SD = 0.034 + 3 × 0.022 OD units) were considered to be positive. All tests were performed in triplicate. The reduction in the mutants' IgE binding was calculated by taking the values obtained for rCuc m 2 (5 µg/ml) as the reference level of 100% allergenic activity for each serum sample.

ELISA inhibition assays were carried out by the same method, except that the pool of sera was previously incubated with 10, 5, 1, 0.1, 0.01, and 0.0001 µg/ml of wild type-rCuc m 2, Mut 1, or Mut 2 as inhibitors (3 h at 25 °C). All tests were performed in triplicate.

2.7. Basophil activation test (BAT)

Wild type rCuc m 2, Mut 1 and Mut 2 were tested by performing the BAT with eight whole blood samples from melon-allergic donors (Table 1, patients 1–8) and two controls. BAT was performed as previously described (Gamboa et al., 2007; Sanz et al., 2002). Following separation of blood cells, 50 µl of the patient's cell suspension was incubated with 50 µl of three final concentrations of the tested samples: 1, 0.1 and 0.01 µg/ml for native rCuc m 2, Mut

1, Mut 2, and Pho d 2, with the latter used as a profilin positive control. As a positive control, a monoclonal anti-IgE receptor antibody (Bühlmann, Allschwil, Switzerland) was used at a final concentration of 1 µg/ml. The test was considered positive when the SI (calculated as the percentage of activated basophils with allergen to the percentage of activated basophils at baseline conditions) was ≥ 2.5. Only tests with positive response to Cuc m 2 were taking into account (patient 1 and 6 were excluded for this test).

2.8. Skin prick test (SPT)

Skin prick tests with the purified proteins wild type rCuc m 2, Mut 1 and Mut 2 were performed by dissolving proteins in glycerol:PBS (v:v) at 50 µg/ml (EAACI, Standardization SoSTaA, 1989). Histamine dihydrochloride (10 µg/ml) and PBS buffer/glycerol solution served as positive and negative controls, respectively. Six tolerant volunteers without symptoms and who had negative IgE and SPT responses to profilin were tested as a control group.

2.9. T cell proliferation assays

T cell lines (TCL) with specificity for Cuc m 2 were established from PBMCs of melon allergic patients as described elsewhere (Tordesillas et al., 2009). Wild type rCuc m 2 or each mutant (1 µg/ml) was added to the culture with specific-TCLs (10⁵ cells per well) and their mitomycin-treated PBMCs (10⁵ cells per well) in triplicate 96 wells for 48 h. During the last 16 h, [³H]-thymidine (0.5 µCi/well) was added, and the incorporated radioactivity was measured by scintillation counting. Before counting, supernatants were recovered to quantify the cytokines (IL4, IL10 and IFNγ) by ELISA.

The optimal concentration of tested proteins was determined by preliminary dose–response experiments using PBMCs from three patients and three concentrations of wild type and the mutants (10, 5 and 1 µg/ml). The concentration of 1 µg/ml proved to be the most appropriate for performing the assays. Phytohaemagglutinin-L from *Phaseolus vulgaris* (PHA; 1 µg/ml; Roche, Mannheim, Germany) was used as a positive control to evaluate the proliferative response of TCL cultures. The stimulation index (SI) was calculated as the ratio of counts of antigen-stimulated cultures to those without any activator.

2.10. HLA class II blocking assays

Cuc m 2-TCLs (10⁵ cells per well) and their mitomycin-treated PBMCs (10⁵ cells per well) were seeded in 96 wells as previously described (Tordesillas et al., 2009). They were incubated with 0.5 and 5 µg/ml of anti-HLA-DP/DQ/DR (AbSerotec, Dusseldorf, Germany) or isotype control antibodies (Alpha Diagnostic International Inc., San Antonio, TX, USA) for 2 h at 37 °C before addition of Cuc m 2 or its mutants and testing of the proliferative response.

2.11. Determination of IL4, IL10 and IFNγ levels in TCL cultures

Cytokine levels were quantified using 50 µl of TCL supernatants using matched antibody-pairs according to the manufacturer's instructions (sensitive limits: IL4, 0.6 pg/ml; IL10, 1.2 pg/ml; IFNγ, 2 pg/ml; ImmunoTools, Friesoythe, Germany). Cultures containing TCLs with mitomycin-treated PBMCs alone served as negative controls.

2.12. Real-time PCR

mRNA was isolated according to the Quiagen-RNeasy protocol (Quiagen, Valencia, CA, USA) and stored at –80 °C. RT-PCR was performed as previously described (Lopez-Torrejon et al., 2005).

cDNA was amplified using Sybergreen mix according to the manufacturer's recommendations and run on a BioRad CFX96 detection system, using previously described primers (Lipscomb et al., 2009; Vale-Pereira et al., 2011). Real-time PCR conditions were as follows: 10 min at 95 °C; 15 s at 95 °C and 45 s at 60 °C (40 cycles). The amount of GATA-3, FoxP3, T-bet and ROR- γ t mRNA expression was normalized with endogenous control EF-1 and the relative quantification was performed using the comparative threshold cycle method ($2^{-\Delta\Delta C_t}$), as described by Livak and Schmittgen (2001). The changes in gene expression were calculated with respect to the untreated cells. All amplifications were carried out in duplicate.

2.13. Statistical analysis

Statistical analysis was performed using SPSS 17.0. Proliferative responses of T cell lines to each protein (wild type and mutants) from patients, cytokine levels and gene expression were compared using the Wilcoxon paired samples test. Values of $p < 0.05$ were considered to be significant.

3. Results

3.1. Exchange of conserved amino acid residues in IgE epitope of Cuc m 2 by site-directed mutagenesis

Based on the IgE conformational epitope recently described in Cuc m 2 (Tordesillas et al., 2010), and the conservation of residues in melon profilin and other members of the same protein family, we generated two variants of Cuc m 2: Mut 1 and Mut 2 (Fig. 1A). The residues conserved in human profilin (PLP1; W₃A₅Y₆L₁₂₂) and included in the epitope were all changed to alanine, except for A₅, which was modified to glycine (Mut 1). In the same way, the remaining residues were mutated in Mut 2 (S₂D₉H₁₀T₁₁₁P₁₁₂G₁₁₃Q₁₁₄N₁₁₆M₁₁₇R₁₂₁). A third mutant, in which all the epitope residues were changed to alanines (except A₅), was also designed. Unfortunately, this mutant could not be expressed in the soluble fractions of *E. coli*.

The computational analysis of folding of the mutant with the greater number of changes, Mut 2, demonstrated that the mutations had little effect on the 3D structure (Fig. 1B). Although the main chains showed slight deviations in some loops between helices and a small shift in N-term ends of the largest helix (Fig. 1B), the superposition of the two structures yielded an RMS of 0.701 Å, increasing only to 0.721 Å upon inclusion of all atoms. Insofar as the mutated amino acids are mostly located in helices, their change modifies the protein architecture very little.

The two Cuc m 2 mutants, Mut 1 and Mut 2, were produced in *E. coli* BL21 (DE) and purified by gel filtration. For the mutants, a retained fraction from poly-L-proline affinity chromatography column could not be obtained (Lopez-Torrejon et al., 2005), due to the partial overlap of the epitope and the poly-L-proline binding site (W₃Y₆I₂₅W₃₃L₁₂₂Y₁₂₅L₁₂₆) (Kaiser and Pollard, 1996; Korupolu et al., 2009).

Purity of recombinant proteins was assessed by SDS-PAGE (Fig. 1C, Coomassie), MALDI analysis (Cuc m 2: 13,968 Da; Mut 1: 13,656.6 Da; Mut 2: 13,624.7 Da) and N-terminal amino acid sequence. Immunoblot analysis showed that no difference between wild type and mutant forms of Cuc m 2 could be detected by the anti-profilin antibody (Fig. 1C, anti-profilin). However, IgE binding of Mut 2 was considerably lower than wild type (Fig. 1C, sera) when the serum pool from melon-allergic patients was used. This reduction indicated that IgE-binding capacity had been destroyed.

A

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Cuc m 2  MSWQAYVDDHLMCDIDGNRLTAAAILGQDGSVWSQSATFPFRLEEIAAILKDFDQPGTL
Mut 1    MSWQAYVDDHLMCDIDGNRLTAAAILGQDGSVWSQSATFPFRLEEIAAILKDFDQPGTL
Mut 2    MSWQAYVDDHLMCDIDGNRLTAAAILGQDGSVWSQSATFPFRLEEIAAILKDFDQPGTL
          * * * * *
Cuc m 2  APTGLFLGGTKYMYIQGEAGAVIRGKKSGGITVKKTNQALIIIGYDEPLTPGQCNMIVE
Mut 1    APTGLFLGGTKYMYIQGEAGAVIRGKKSGGITVKKTNQALIIIGYDEPLTPGQCNMIVE
Mut 2    APTGLFLGGTKYMYIQGEAGAVIRGKKSGGITVKKTNQALIIIGYDEPLTPGQCNMIVE
          * * * * *
Cuc m 2  RLGDYLIEQGL-
Mut 1    RLGDYLIEQGL-
Mut 2    RLGDYLIEQGL-
          * * *
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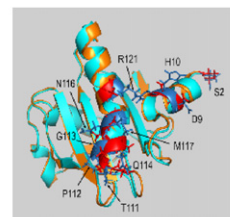
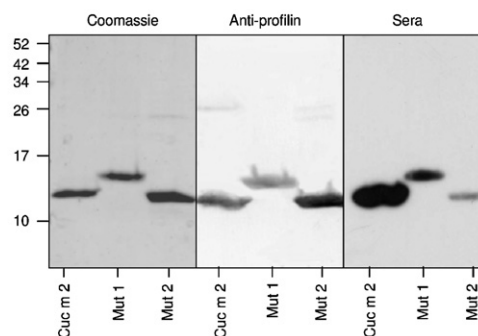
B**C**

Fig. 1. (A) Alignment of amino acid sequences of rCuc m 2 and mutant (Mut 1 and Mut 2) forms. The residues identified in the IgE epitope of Cuc m 2 are shaded in gray and the changed residues in each mutant appear in black. The binding site of poly-L-proline is indicated by an asterisk. (B) Ribbon diagram of superposed optimized structures of Cuc m 2 (cyan) and Mut 2 mutant (orange). Labelled Cuc m 2 residues (blue) changed to alanine (red) in Mut 2 mutant are drawn as sticks. (C) rCuc m 2 and its mutants were separated by SDS-PAGE and stained with Coomassie Blue (Coomassie) or immunodetected with anti-profilin rabbit antibodies (anti-profilin) or with a serum pool from melon-allergic patients (Sera). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. Characterization of the immunogenic activity of wild type and mutant forms of Cuc m 2

The IgE reactivity of seven individual patients' sera (Table 1, patients 7–13) was also evaluated by ELISA (Fig. 2A). All the sera displayed IgE reactivity towards wild type rCuc m 2, although there was considerable variation in the specific IgE level. Using Mut 2, the recognition decreased by 65% with the serum pool from allergic patients, the reduction with the individual sera ranging from 35% to 78%. In contrast, Mut 1 exhibited similar IgE reactivity to wild type rCuc m 2 in most of the allergic patients tested. Thus, a 34% reduction in IgE-binding capacity was observed, varying from 0% to 46% in individual sera.

On the other hand, both mutants attained a maximum inhibition of around 60%. Therefore, they were unable to inhibit the wild type-IgE binding capacity (Fig. 2B).

3.3. Reduced ability of Mut 2 to induce in vivo allergic response

To analyze the biological activity of mutants, basophils from melon-allergic patients were incubated with wild type or mutants

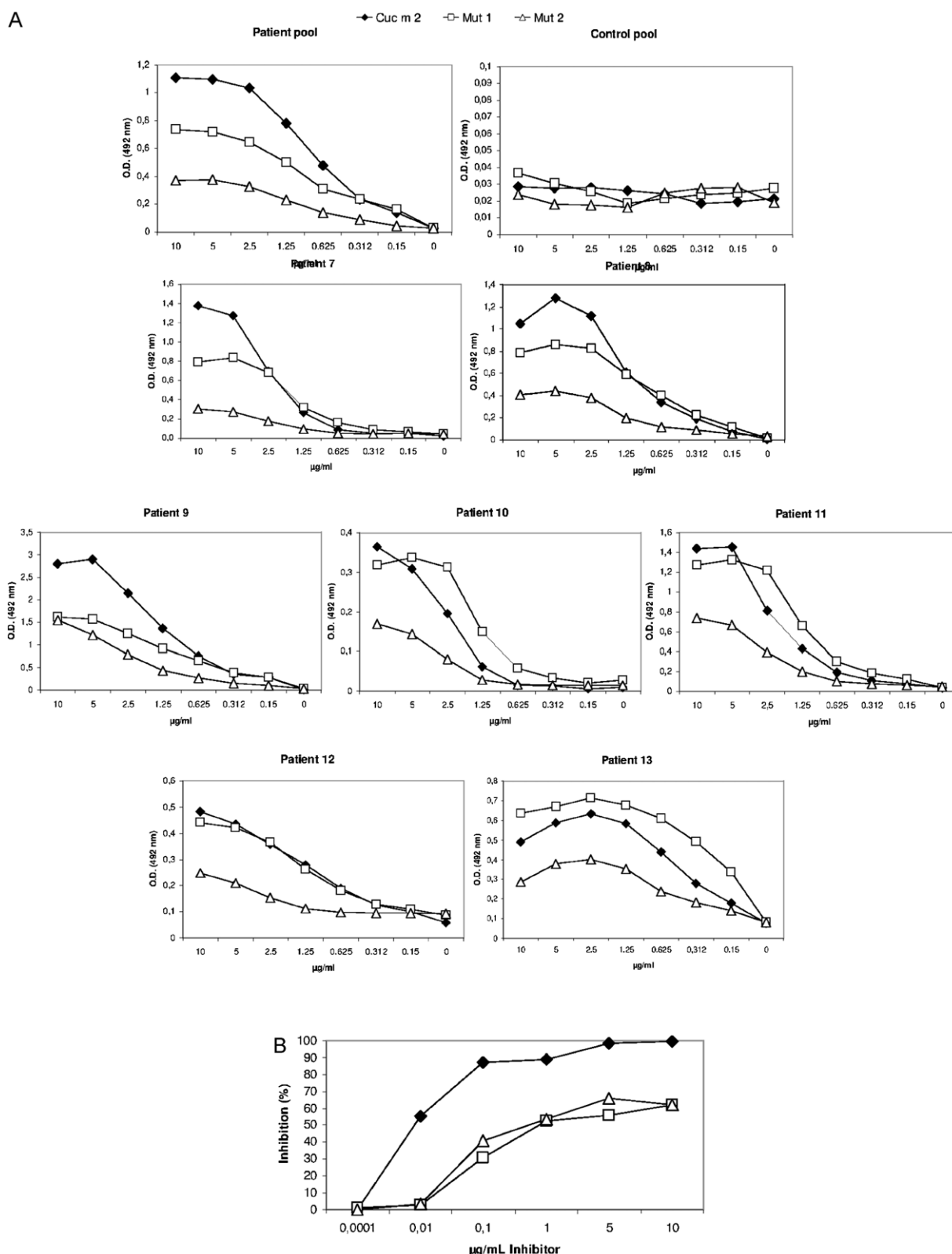


Fig. 2. (A) Specific IgE to rCuc m 2 or its mutants (Mut 1, Mut 2) using sera from melon-allergic patients (individual or pooled) or a control serum pool (1:2 dilution). Results were considered positive for values greater than 0.1 OD units [mean (OD) + 3 SD of negative control]. The number of the patients corresponds to Table 1. (B) ELISA inhibition assays. The inhibition percentage of the IgE-binding capacity of rCuc m 2, Mut 1, and Mut 2 is represented. Plates were covered with rCuc m 2 (5 µg/ml). After blocking, a serum pool obtained from melon-allergic patients was added after previous incubation with 10, 5, 1, 0.1, 0.01, and 0.0001 µg/ml of purified proteins as inhibitors (3 h at 25 °C). All tests were performed in triplicate.

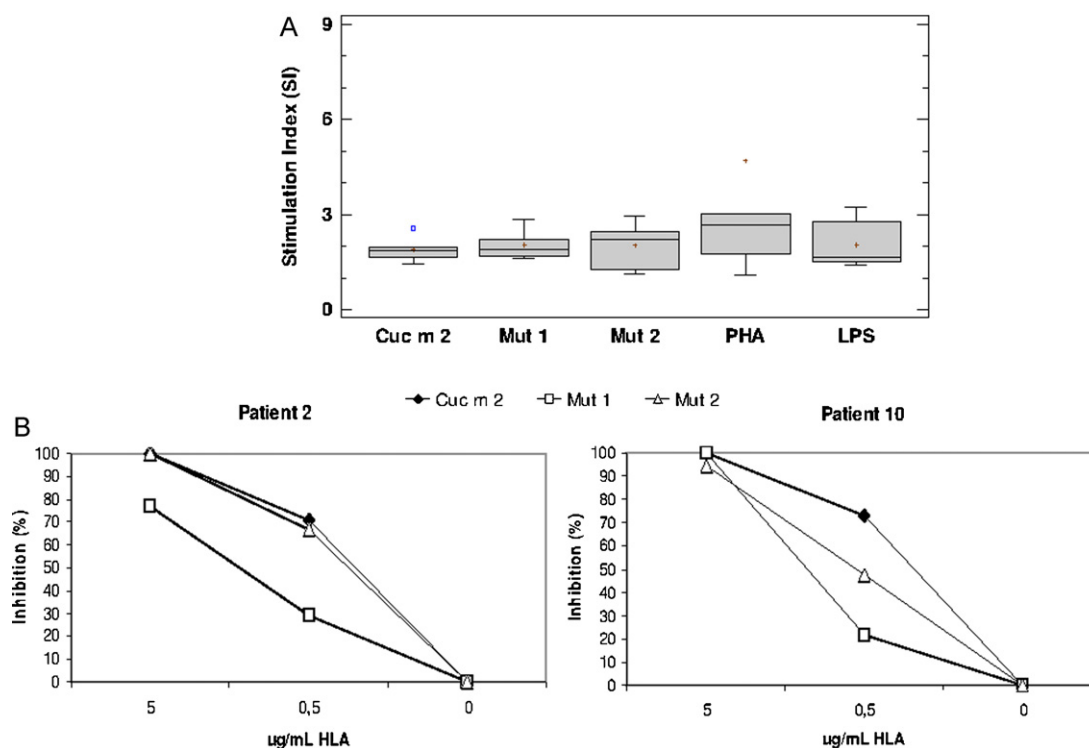


Fig. 3. (A) Stimulation index (SI) of Cuc m 2 T cell lines from melon-allergic patients to the wild type (Cuc m 2) or mutants (Mut 1, Mut 2). Medians (–) and ranges (bars) were calculated from 18 values for each protein. (B) HLA-II restriction specificity of T cell recognition of Cuc m 2 and the mutants. The inhibition percentage of the proliferation of specific TCLs to each protein in the presence of anti-HLA antibodies is represented for two patients. The number of patients corresponds to Table 1.

of Cuc m 2 (Table 1). We observed greatly reduced Mut 2 activity in the basophil activation test, with a positive response in only two out of six patients.

On the other hand, the capacity of wild type rCuc m 2 and the two mutants to elicit skin reactions was evaluated in eight allergic patients by *in vivo* SPT (Table 1). Mut 1 induced wheal reactions comparable to those induced by wild type. By contrast, Mut 2 also had a reduced capacity to induce allergic skin reactions, being recognized by only four of the eight patients tested.

Curiously, some patients (2 and 5) showed a different response to the same protein by BAT and SPT. This controversy could be associated with different cellular mechanisms involved in the two tests.

3.4. Purified wild type and mutant forms of Cuc m 2 induce similar proliferative responses in specific T cell lines

T cell lines (TCLs) from six melon-allergic patients were selected with rCuc m 2. The culture of these T cell lines in the presence of wild type or mutants of Cuc m 2 induced cellular proliferation (Fig. 3A). This proliferation was prevented by incubation with anti-HLA antibody in a dose-dependent manner (Fig. 3B). Isotype control antibody had no effect (data not shown).

Wild type and mutants of Cuc m 2-induced proliferation showed significant differences from the untreated culture (Fig. 3A; Wilcoxon test, $p = 0.028$ for the three antigens). Values of the stimulation index obtained for rCuc m 2 were of the same magnitude as those obtained with Mut 1 and Mut 2 (Fig. 3A, Wilcoxon test: Cuc m 2 versus Mut 1, $p = 0.080$; Cuc m 2 versus Mut 2, $p = 0.753$). These results suggest that the mutants contained the corresponding T cell epitopes of Cuc m 2. Mutations introduced in Mut 1 and Mut 2 did not affect T cell recognition, inducing proliferative responses comparable to those of wild type form.

Furthermore, in response to the three proteins, IL4 was produced in a significantly different way by Cuc m 2 T cell lines with respect to non-treated (Fig. 4A). By contrast, no differences were found in the content of IL10 and INF γ between the treated and untreated cell lines (Fig. 4A).

The expression of the transcription factors for Th1, Th2, Treg and Th17, T-bet, GATA-3, Fox p 3 and ROR γ t respectively, was measured by RT-qPCR (Fig. 4B). The highest level of expression was obtained for GATA-3 and T-bet. The expression levels were similar in the wild type form and the mutants, with no statistically significant differences except for Mut 1 in GATA-3 and Fox p 3, in which the level of expression were lower.

4. Discussion

Melon profilin is highly prevalent in melon-allergic patients (80%), who usually suffer from pollinosis. This co-sensitization may result in cross-reactivity among members of the profilin family, as described elsewhere (Lopez-Torrejon et al., 2005; Radauer, 2004). These patients do not often respond to conventional immunotherapy with extracts where profilin is poorly represented. Thus, the production of hypoallergenic profilins might be a good tool to develop specific immunotherapy. In a previous study, our group used phage display technology to identify melon-Cuc m 2-IgE-conformational epitopes (Tordesillas et al., 2010). The IgE epitope was defined and mapped onto the Cuc m 2 surface to yield the sequence S₂W₃A₅Y₆D₉H₁₀T₁₁P₁₂G₁₁₃Q₁₁₄N₁₁₆M₁₁₇R₁₂₁L₁₂₂. This epitope was conserved in other pollen profilins such as timothy grass-Phl p 12 and birch-Bet v 2, thus suggesting that this region could be involved in cross-reactivity (Barber et al., 2008; Cuesta-Herranz et al., 2010; Pastor et al., 2009; Radauer et al., 2006).

In this study, we produced two mutants (Mut 1 and Mut 2) of Cuc m 2, the major melon allergen, on the basis of the previously identified epitope (Tordesillas et al., 2010). The 3D modelling and

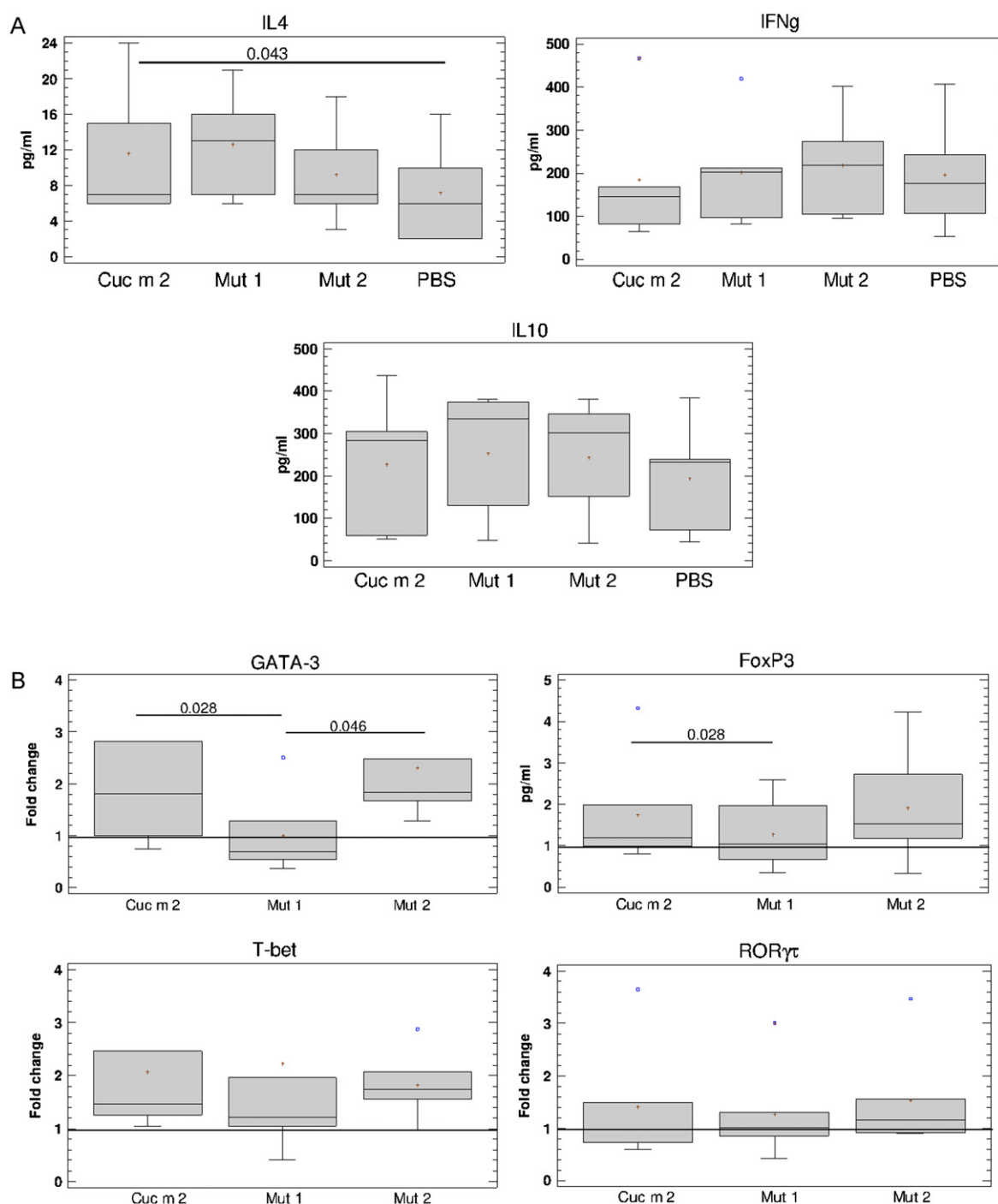


Fig. 4. (A) T-cell lines cytokine production is represented ($n=6$). Statistical significance is indicated after performing a paired-samples Wilcoxon test ($p<0.05$). Medians (–) and ranges (bars) are shown. (B) GATA-3, FoxP3, T-bet and RORγt gene expression levels for T cell lines from melon-allergic patients ($n=6$). The results are expressed as n -fold difference between non-stimulated with stimulated conditions. Statistical significance is indicated (Wilcoxon test; $p<0.05$). Medians (–) and ranges (bars) are also shown.

analysis of the potential surface demonstrated that the wild type and mutants had similar folding. In Mut 1, only four residues of this epitope were changed, and these amino acids did not seem to be relevant to the allergenic activity of melon profilin in most patients. Mut 1 had similar allergenic activity to wild type form, as measured by BAT and SPT. In fact, in patient 8, the Mut 1 response was higher than that obtained with wild type, in both tests. This can be due to the polyclonal response of IgE. However, in immunological *in vitro* assays, several patients (3/7) showed a slightly reduced response to Mut 1 compared to wild type (32–46% reduction).

In the case of Mut 2, the main residues of the IgE conformational epitope were changed. The allergenic activity of Mut 2 was considerably lower (by up to 78%) than the one obtained by wild type form in ELISA assays. The lower IgE reactivity of Mut 2 also resulted in reduction of their allergenic activity. Using basophil activation and skin prick tests in melon-allergic patients, we found that Mut 2 exhibited reduced biological activity. Such results suggest that these residues are important in Cuc m 2-allergenic activity. For this reason, the IgE conformational epitope described previously by means of phage

display seems likely to be an important IgE epitope of this allergen.

Curiously, in two patients, the response obtained in BAT and SPT were different. These results could be explained because two different mechanisms were involved. Therefore, although generally the correlation between both tests is high, there can be differences (de Weck et al., 2008; Erdmann et al., 2003; Sanz et al., 2001).

Mut 2 described in the present study exhibited reduced allergenic activity but maintained the ability to induce lymphoproliferative responses in Cuc m 2-specific T cell lines. It may thus be expected that Mut 2 could also be used for the treatment of allergies to Cuc m 2-related allergens from other profilin sources. Surprisingly, T cell epitopes must be unaltered in Mut 2 since it does not lose its capacity to induce T cell proliferation at the same level as does the wild type and the cytokine profile remains unaltered. The proliferation was specific as it can be determined by the inhibition assays with anti-HLA. Thus, the values obtained for Cuc m 2 and the mutants confirmed the preservation of the T cell epitopes. The analysis of the expression of gene markers, such as GATA-3, T-bet, Foxp3, and ROR γ t, showed no differences among wild type and mutants, except in Mut 1 for GATA-3 and Foxp3. In immunotherapy, it is essential that T epitopes remain functional (Till et al., 2004). For this reason, Mut 2 would be an ideal candidate for use in improving immunotherapy, although further analysis would be necessary.

In conclusion, we have produced a hypoallergenic mutant of Cuc m 2 that did not affect the T cell epitopes. The residues changed in Mut 2 are essential for IgE binding, both *in vitro* and *in vivo*. This mutant shows promise for immunotherapy in patients who are allergic to profilins and may also avoid putative cross-reactions among pollen and foods.

Conflicts of interest

Authors have no conflicts of interest.

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