Engineering of hypoallergenic mutants of the *Brassica* pollen allergen, Bra r 1, for immunotherapy

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Abstract The *Brassica* pollen allergen Bra r 1 belongs to a new family of Ca^{2+}-binding proteins, characterized by the presence of two potential EF-hand calcium-binding domains. Disruption of these EF-hand motifs by amino acid substitutions demonstrated that both domains of Bra r 1 constitute functional Ca^{2+}-binding sites. Calcium-binding deficient mutants displayed significantly reduced IgE-binding activity. Injection of these mutated Bra r 1 variants into a murine model system showed that mouse IgG raised against the mutants recognized native Bra r 1 in *Brassica* pollen extracts suggesting the potential use of the engineered allergens for effective immunotherapy.

Key words: *Brassica rapa*; Ca^{2+} binding; IgE reactivity; Mutagenesis analysis; Pollen allergen; Recombinant protein

1. Introduction

Up to 20% of the human population in developed countries suffer from IgE-mediated atopic diseases such as allergic rhinitis, conjunctivitis and bronchial asthma. Pollen from trees, weeds, grasses and other plants represents the major airborne cause of these allergic disorders. Diagnosis and treatment of allergic patients require the production of large amounts of pure and well defined allergens. Recombinant DNA technology has proven to be a useful tool for the isolation and characterization allergenic proteins. Many cDNA clones encoding major and minor pollen allergens have been isolated from several plant species and recombinant proteins have been used for determination of epitopes, diagnosis and immunotherapy [1–5].

Pollen allergens have been classified into different groups according to sequence homologies and immunological properties [6]. Recently, a novel class of Ca^{2+}-binding pollen allergens has been identified in such diverse plant species as *Brassica rapa* Bra r 1 [7]; Bermuda grass Cyn d 7 [8,9]; birch Bet v 4 [10,11]; and olive Ole e 3 [12]. This group of proteins is characterized by the presence of two potential Ca^{2+}-binding sites of the EF-hand type. Members of this class of allergens are small, water soluble proteins of about 9 kDa that share significant sequence similarities extending outside the Ca^{2+}-binding domains. Furthermore, it has been shown that Bra r 1, Cyn d 7 and Bet v 4 share significant IgE cross-reactivities [8,9] and must thus be considered as relevant cross-reactive plant allergens.

With the aim to develop an allergen-specific immunotherapy, some studies have been carried out to design mutant allergens which display low/no IgE-binding activity, but still retain T cell epitopes and thus T cell activating capacity, as demonstrated for the major house dust mite allergen [13,14]. Such molecules would be of significant importance for novel approaches in immunotherapy, since they substantially decrease the risk of IgE-mediated anaphylactic side effects. Engineering and precise characterization of mutant proteins with reduced IgE-binding activity is a prerequisite for future application in immunotherapy.

In this report, we characterized native Bra r 1 in *Brassica* pollen extracts and investigated the effect of protein-bound calcium on IgE binding properties of recombinant Bra r 1. We also generated point mutations in the first, and the second EF-hand motifs individually and in both motifs together which led to disruption of the Ca^{2+}-binding domains. These Bra r 1 mutants showed significantly reduced IgE-binding capacities. To evaluate the potential use of Bra r 1 mutants for specific immunotherapy, mutant proteins were injected into mice and antisera were analyzed for IgG reactivity to native Bra r 1.

2. Material and methods

2.1. Plant materials and protein extracts

Brassica rapa L. plants were grown under greenhouse conditions and pollen grains were collected. Pollen extracellular protein was extracted as described in Evans et al. [15]. Mature pollen grains of *B. rapa* were delipidated with cold acetone. Extracellular proteins were extracted by vigorously vortexing for 10 min in 0.1 M Tris-HCl (pH 8.0) buffer. The supernatants were collected and used for immunoblot analysis.

2.2. Construction of expression plasmid for mutated Bra r 1

Internal amino acid substitutions in two calcium-binding sites were engineered by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Oligonucleotide primers to construct Bra r 1 mutants by PCR are listed as follows: primer 1: TTGACACTGCCGGCGCTGCTAAAATATCAGC; primer 2: GCTGATATTTTAGCAGCGCCGGCAGTGTCAA; primer 3: GATACTGATGGTGCTGCAAACAAATCGTTTCAGG; and primer 4: CCTGAAACGATTTGTTTGCAGCACCATCAGTATC. Bases exchanged are underlined. The wild-type Bra r 1 cDNA clone in pBluescript II SK$^-$ [7] was used as a template. Primers 1 and 2 were used for destruction of EF-hand 1 of Bra r 1 by replacing Asp^{16}, Asp^{18} and Gly^{19} by Ala (mu1), and primers 3 and 4 for EF-hand 2 by replacing Asp^{53} by Ala, Gly^{54} by Ala and Ile^{56} by Lys (mu2). Residues are numbered from the first Met of Bra r 1. The sequences of the mutated clones were confirmed. Wild-type Bra r 1 (WT), mu1 and mu2 were excised from pBluescript II SK$^-$ with *Bam*HI and *Sac*I and subcloned in the expression plasmid, pQE-30 (Qiagen). The double mutant (muW), which contained two defective EF-hands, was constructed by replacing the EF-hand 1 region of mu2 with the mutated EF-hand 1 of mu1.

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2.3. Expression and purification of recombinant Bra r 1

Wild-type Bra r 1 and the three mutants cloned into the expression vector pQE-30 were introduced into *E. coli* M15 cells. Expression of recombinant proteins was induced by addition of isopropyl-β-D-thiogalactopyranoside, and cells were harvested after 5 h of induction. According to the manufacturer's instructions (Qiagen), recombinant proteins were purified by nickel-nitrilotriacetic acid metal affinity under denaturing conditions, using urea buffer. The denatured proteins were refolded by dialysis against PBS buffer (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4) and protein concentrations were determined using the Bio-Rad assay (Bio-Rad, CA, USA).

2.4. $^{45}Ca^{2+}$ overlay assay

[^{45}Ca]Cl_2 (5–50 mCi/mg Ca^{2+}) was purchased from Amersham. Equal amounts of recombinant proteins of Bra r 1 variants were separated by electrophoresis on 15% SDS-polyacrylamide gels. After electrophoretic transfer to PVDF membranes (PVDF-Plus; Micron Separations), blots were washed with the buffer (60 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole, pH 6.8), and then overlaid with the same buffer containing 1 μCi/ml [^{45}Ca]Cl_2 (1.31 μM $CaCl_2$) for 10 min. Subsequently, the blot was washed with distilled water and 50% ethanol, air-dried, and autoradiographed as described [16].

2.5. Antibody production

Antibodies against entire recombinant Bra r 1 (anti-rBra r 1) were raised in mice. An expression plasmid was constructed by inserting Bra r 1 cDNA into PinPoint Xa-3 vector as previously reported [7]. Recombinant fusion protein of Bra r 1 was expressed in *E. coli* XL-1 blue and purified using PinPoint Protein Purification System (Promega). Bra r 1 fusion proteins were injected with complete Freund's adjuvant (Difco) intraperitoneally into BALB/c mice and antiserum was collected. Antiserum was purified to obtein anti-rBra r 1 specific IgG as described in Lin et al. [17]. The specificity of anti-rBra r 1 antiserum was tested by immunoblot analysis, using pollen extracts of *B. rapa*.

Antisera against recombinant mutant Bra r 1s were prepared by injecting affinity-purified mutant proteins (30–50 μg) with complete and incomplete Freund's adjuvant (Sigma) subcutaneously into BALB/c mice at intervals of two weeks. Blood samples were taken one week after the immunizations and production of specific IgG antibodies against mutant proteins was confirmed by immunoblot analysis.

To obtain anti-peptide antiserum (anti-C-pep), a peptide of 15 amino acids corresponding to the C-terminal region of Bra r 1 (ASANPGLMKDVAKVF) was synthesized and injected into rabbits (Sawady technology). Antiserum was collected and used for immunoblot and ELISA assay.

2.6. Immunoblot analysis

Recombinant proteins and *Brassica* pollen extracts were separated by SDS-PAGE (15% polyacrylamide gel) or thin-layer polyacrylamide gel isoelectric focusing (IEF; p*I* 3.5–9.5, Pharmacia). After electrophoresis, proteins were electroblotted onto PVDF membranes (PVDF-Plus, Micron Separations; and Immobilon, Millipore). The blots were probed with mouse anti-rBra r 1, rabbit anti-C-pep or sera from allergic individuals. After washing with TBS or PBS buffer containing 0.05% Tween-20, the blots were incubated with secondary antibody, alkaline phosphatase (AP)-conjugated anti-mouse IgG (Promega), AP-conjugated anti-rabbit IgG (Promega), AP-conjugated anti-human IgE antibodies (Sigma), or peroxidase-conjugated anti-human IgE (KPL). After washing, the signal was visualized by incubation with 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt and nitroblue tetrazolium chloride solution for AP or using enhanced chemiluminescence for peroxidase according to the ECL Western Blotting Analysis System (Amersham).

2.7. ELISA

Antibody binding capacity of recombinant Bra r 1 (rBra r 1) was determined by ELISA. ELISA and inhibition ELISA were performed as previously described [18]. Microtiter plates (Greiner Labortech) were coated with 50 ng/well rBra r 1. The bound antigen was washed with PBS containing 0.05% Tween-20 (PBST) and the free sites were blocked with 1% BSA in PBST. Subsequently plates were incubated overnight with either 1:10 diluted human sera, 1:500 diluted mouse anti-rBra r 1 or 1:10 000 diluted rabbit anti-C-pep in 1% BSA in PBS at 4°C. After washing with PBST, the plates were incubated with 1:4000 diluted AP-conjugated anti-human IgE (Sigma), 1:7500 diluted AP-conjugated anti-mouse IgG (Promega), or 1:5000 diluted AP-conjugated anti-rabbit IgG (Promega) at room temperature. After washing, ELISA substrate (KPL) was added. The reaction was stopped by addition of 2.5% EDTA and optical density was measured at 630 nm, using a plate reader (Packard). For ELISA inhibition analysis, primary antibodies were preincubated with different concentrations of Bra r 1 variants (10–1000 ng/ml). The percentage of inhibition was calculated using the following formula: inhibition (%) = $100 \times [(A-B)/A]$, where A represents absorbance without the inhibitor and B absorbance with inhibitor. Triplicate assays were performed for each dilution and average values are displayed.

3. Results

3.1. IgE reactivity of Bra r 1

Bra r 1 has been isolated as a cDNA clone encoding a pollen allergen of *Brassica rapa* by IgE-immunoscreening of an anther cDNA expression library [7]. However, Bra r 1 protein has not yet been identified in *Brassica* pollen extracts.

In order to identify Bra r 1 among the proteins present in a pollen extract with patient's serum, the following competition experiment was carried out: rBra r 1 produced in *E. coli* M15 as a fusion protein with a histidine tag at the N-terminus, was affinity purified and used for depletion of antibodies specific to Bra r 1. The serum used in this study reacted with several proteins in an IEF-gel blot of extracellular *Brassica* pollen proteins (Fig. 1, lane 1). Preincubation of the serum with rBra r 1 revealed loss of a band at p*I* 4.34 (Fig. 1, lane 2; arrow head), indicating that the band at p*I* 4.34 represents Bra r 1 protein. This p*I* value coincides with that predicted from the cDNA sequence of Bra r 1.

For further analysis of Bra r 1 protein, a mouse antibody

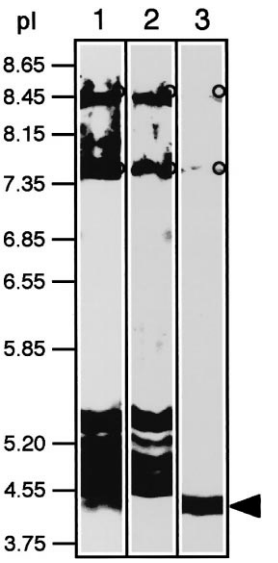

Fig. 1. Identification of a *Brassica* pollen allergen encoded by Bra r 1. Soluble pollen extract of *B. rapa* was separated by IEF and the blots were probed with patient's serum (lane 1), or patient's serum preincubated with rBra r 1 (lane 2). IgE binding was detected by ECL. The membrane strip shown in lane 2 was reprobed with mouse specific antibody against rBra r 1 (lane 3). Arrow head represents the position of Bra r 1. Circles indicate the position of sample application on a piece of filter paper and do not indicate the signals.

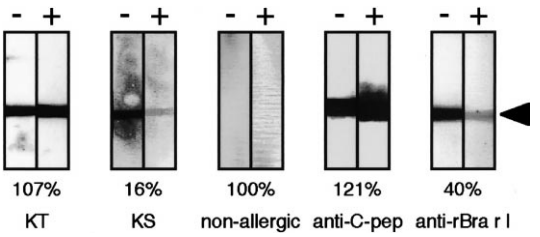

Fig. 2. Effect of protein-bound Ca^{2+} on antibody binding of rBra r 1. Recombinant wild-type Bra r 1 was separated by SDS-PAGE, blotted onto PVDF membranes and tested for antibody reactivity using sera from pollen allergic patients (KT and KS), serum from a non-allergic individual, rabbit antiserum against a C-terminal polypeptide (anti-C-pep) of Bra r 1, and mouse anti-rBra r 1 antiserum (anti-rBra r 1). Blocking, incubation of primary antibody and washing procedures were carried out either in the presence (+) or absence (−) of 5 mM EGTA. Microtiter plates were coated with rBra r 1 and ELISA assays were performed in the same manner as mentioned above. The percentage of IgE- or IgG-binding activity observed in the absence of EGTA as compared to the reactivity obtained in the presence of EGTA, is given under the immunoblots.

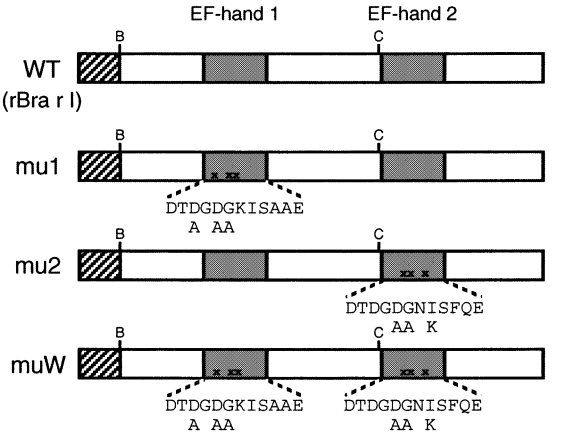

Fig. 3. Schematic representation of the structure of Bra r 1 mutants. Hatched boxes and shaded boxes represent histidine tags and EF-hand motifs, respectively. Amino acid sequences of EF-hand domains are given below the structures. Cross marks in the EF-hand domains indicate the positions of the point mutations. WT: wild-type Bra r 1 fused with histidine tag; mu1: Bra r 1 mutated in EF-hand 1; mu2: mutation in EF-hand 2; muW: mutation in both EF-hands. *Bam*HI and *Cla*I sites used to construct the double mutant are indicated as B and C, respectively.

was raised against rBra r 1. In an IEF-gel blot of extracellular pollen proteins mouse anti-rBra r 1 antibody reacted with a single band at p*I* 4.34. This result corresponded with the data obtained in the depletion experiment of human IgE (Fig. 1, lane 3). Thus, it is clear that among the proteins present in a pollen extract mouse anti-rBra r 1 antibody specifically reacted with Bra r 1 exclusively.

3.2. *Effect of Ca^{2+} on IgE reactivity*

As a next step we investigated the influence of protein bound calcium on IgE recognition of rBra r 1 by depleting calcium binding with EGTA. First, sera of 80 pollen allergic patients were tested for their IgE reactivity to rBra r 1 and among them the two sera (KT and KS) that showed strongest reactivity were chosen for the described depletion experiments.

When SDS-gel blots of rBra r 1 were incubated with serum KT, KT IgE bound rBra r 1 in the presence of EGTA with the same intensity as in the absence of EGTA (Fig. 2). This result was also confirmed in ELISA experiments and it was concluded that IgE-recognition of rBra r 1 by KT serum was not influenced by calcium depletion. On the other hand, IgE binding of serum KS was greatly reduced by EGTA treatment (Fig. 2). Quantitative analysis using ELISA assays revealed a reduction of 84%. These data show that the effect of depletion of protein-bound calcium with EGTA varies among patients. Serum IgE from a non-allergic patient did not bind to rBra r 1 in the presence or absence of EGTA (Fig. 2).

We further tested the effect of calcium depletion on the binding of mouse anti-rBra r 1 IgG and rabbit anti-C-pep IgG. In the case of anti-rBra r 1 IgG, depletion of calcium with EGTA showed a substantial reduction of binding to rBra r 1 (Fig. 2), as observed in the case of serum KS. When SDS-gel blots were incubated with anti-C-pep IgG, rBra r 1 was detected equally in the presence or in the absence of EGTA, indicating that removing Ca^{2+} had no effect on the structure of the C-terminal epitope of rBra r 1 (Fig. 2).

3.3. *Calcium binding ability of Bra r 1 mutants*

To evaluate whether both EF-hand domains are able to bind calcium, we introduced point mutations in each domain. The introduced mutations are summarized in Fig. 3. Mu1 contains three point mutations in domain 1, mu2 contains three point mutations in domain 2, and muW contains both mutated domains. Each recombinant protein was expressed in

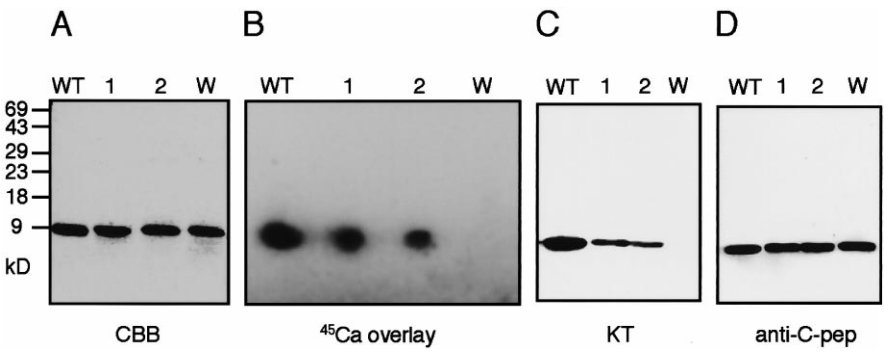

Fig. 4. Calcium-binding ability and immunoblot analysis of Bra r 1 mutants. Affinity-purified recombinant proteins of wild-type Bra r 1 (WT) and three Ca^{2+}-binding deficient mutants of Bra r 1 (mu1, mu2 and muW) were separated by SDS-PAGE. A: Coomassie Blue-stained gel. B: An autoradiogram of a blot overlaid with $[^{45}Ca]Cl_2$ solution. Protein blots were probed with sera from patient KT (C) and rabbit anti-C-pep (D). Molecular weight markers are indicated on the left in A.

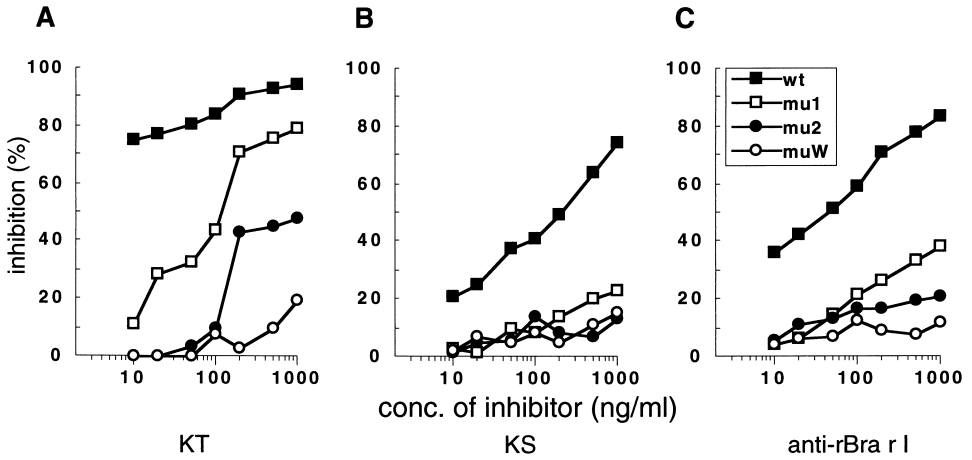

Fig. 5. Inhibition of antibody binding to immobilized rBra r 1 using Bra r 1 variants. Antibody binding to rBra r 1 was inhibited by preincubation of sera from patient KT (A), patient KS (B), and anti-rBra r 1 (C) with increasing concentration of wild-type Bra r 1 (filled square), mu1 (open square), mu2 (filled circle) and muW (open circle).

E. coli as a fusion protein with an N-terminal histidine tag, purified and analyzed by SDS-PAGE.

Coomassie Brilliant Blue staining of an SDS-gel showed that each recombinant protein was properly expressed with the expected molecular size of 9 kDa (Fig. 4A), and confirmed that equal amounts of proteins were loaded. $^{45}Ca^{2+}$ overlay assay showed a strong signal in wild-type Bra r 1 (Fig. 4B). The mutants, mu1 and mu2, each containing one intact calcium-binding domain, clearly bound calcium, although the signal was approximately 50% of that of wild-type Bra r 1 (Fig. 4B). The double mutant, muW, which had both calcium-binding sites disrupted, showed a complete loss of calcium-binding activity (Fig. 4B). These results indicate that both domains have the ability to bind calcium and that site-directed mutagenesis successfully abolished the calcium-binding ability of each domain.

3.4. Reactivities of IgE to Bra r 1 mutants

IgE-binding capacity of recombinant mutant Bra r 1s was determined by ELISA inhibition analysis. Patients' sera preincubated with different amounts of rBra r 1 variants were added to ELISA plate wells coated with wild-type rBra r 1.

The ability of the mutant proteins to inhibit binding of IgE to rBra r 1 was shown as percent inhibition in Fig. 5. In the case of KT serum, wild-type Bra r 1 gave 94% inhibition, mu1 79%, mu2 50%, and muW 19% at the highest inhibitor concentrations tested (1000 ng/ml). In the case of KS serum, percent inhibition of IgE binding was remarkably lower in mu1, mu2 and muW than in the wild type. At the highest concentration of inhibitors (1000 ng/ml), 74% inhibition was observed with wild-type Bra r 1, 23% with mu1, 16% with mu2, and 13% with muW. These results indicate that KT and KS IgE antibodies have reduced affinity to Bra r 1 mutants, especially domain 2 mutant and the double mutant as compared to the wild-type protein. Mouse IgGs showed profiles of percent inhibition similar to that of KS IgEs (Fig. 5C). These results indicate that mutations in either EF-hand domain significantly reduce IgE- and IgG-binding ability. However, mutation of domain 2 is more effective than mutation of domain 1 in reducing IgE and IgG binding.

The ability of the Bra r 1 mutants to interact with IgE was also examined by SDS-gel blot analysis. IgE from patient KT reacted with mu1 and mu2, but the signals were much weaker than the signal obtained with wild-type Bra r 1 protein and mu2 bound significantly less IgE than mu1 (Fig. 4C). These data are consistent with the results obtained in ELISA inhibition assays. Furthermore, KT serum did not react with muW. Thus, it was concluded that disruption of both Ca^{2+}-binding sites completely abolished IgE binding. The same SDS-blot

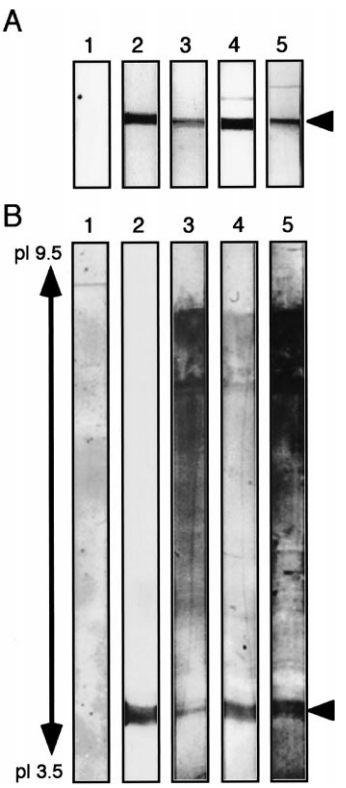

Fig. 6. Immunoblot analysis of recombinant Bra r 1 (A) and *Brassica* pollen extracts (B). Recombinant Bra r 1 and pollen extracts were separated by SDS-PAGE and IEF, respectively, and blotted onto PVDF membranes. Membrane strips were incubated with mouse preimmue serum (lane 1), anti-rBra r 1 (lane 2), anti-mu1 (lane 3), anti-mu2 (lane 4) or anti-muW (lane 5) antisera. Binding IgG was detected by color development. Arrow heads indicate the position of Bra r 1.

was also incubated with anti-C-pep antibody, and identical intensity of bands was observed in case of wild-type Bra r 1 and the mutated proteins (Fig. 4D), indicating that the C-terminal epitope was not affected by disruption of the calcium-binding sites.

3.5. Mouse IgG raised against Bra r 1 mutants recognized native Bra r 1

To evaluate the application of Bra r 1 mutants for allergen-specific immunotherapy, we obtained mouse antisera raised against the mutant proteins mu1, mu2 and muW. The production of specific IgG against the respective mutants was confirmed by SDS-gel blot analysis (data not shown). As shown in Fig. 6A, sera raised against Bra r 1 mutants were still able to react with rBra r 1. Furthermore, each serum also recognized native Bra r 1, as shown in the IEF-gel blot of *Brassica* pollen extract (Fig. 6B).

4. Discussion

Bra r 1 was initially isolated as a cDNA clone encoding a two EF-hand Ca^{2+}-binding pollen allergen by immunoscreening of a *Brassica* pollen expression library with patient's serum [7]. Bra r 1 has an expected small molecular mass of 8.9 kDa and a p*I* value of 4.35. By using an antibody directed against rBra r 1 we identified Bra r 1 protein in a *Brassica* pollen extract with the expected p*I* value (Fig. 1).

Homologous two EF-hand calcium-binding pollen allergens with similar molecular mass and p*I* value have been reported as Ole 3 from olive [12], Bet v 4 from birch [10,11] and Cyn d 7 from Bermuda grass [8,9]. The close similarity of these allergens of very distantly related plant species indicates that they might represent a group of highly conserved pollen allergens. In addition, IgE cross-reactivity that has been observed between Bet v 4, Cyn d 7 and Bra r 1 [9–11] further suggests that this group of Ca^{2+}-binding proteins must be considered as important cross-reactive plant allergens.

Calcium-binding proteins are known to change their conformations in the presence or absence of Ca^{2+} [19–21]. Experiments using EGTA to deplete protein bound calcium indicated that the influence of calcium binding on IgE reactivity towards Bra r 1 varied among patients (Fig. 2). Previous studies showed that IgE recognition of Cyn d 7, Bet v 4 and Bet v 3, a Ca^{2+}-binding pollen allergen with three EF-hand domains, required protein-bound calcium [8,9,11,22]. However, recently Engel et al. [10] reported that two out of five patients' sera did not need calcium for IgE recognition of Bet v 4. Similar results have been reported for parvalbumin, a 12-kDa Ca^{2+}-binding protein, which has been characterized as the major fish allergen where depletion of Ca^{2+} led to a reduction of IgE binding in some patients [23–25]. However, in the case of other patients depletion of Ca^{2+} did not have an effect on IgE reactivity [25].

In our study, in vitro mutagenesis experiments demonstrated that both EF-hand domains constitute functional Ca^{2+}-binding sites (Fig. 4B), since mutations introduced in mu1 and mu2 abolished the binding of calcium (Fig. 4B). Furthermore, we could show that disruption of the two calcium-binding sites resulted in decreased IgE binding to Bra r 1 for both sera tested (Fig. 5B). For KS serum, the observed IgE reactivity patterns were consistent with those obtained upon depletion of protein-bound calcium by EGTA. It was therefore concluded that mutations in the EF-hand domains led to conformational changes in discontinuous IgE epitopes substantial for KS IgE binding (Fig. 2). In addition, it seems that certain amino acids of the EF-hands are also part of continous (linear) IgE epitopes, recognized by IgE antibodies of KT serum. These epitopes are not affected by Ca^{2+} removal mediated local conformational changes. Nevertheless, these linear epitopes also seem to be destroyed by amino acid substitutions, since KT IgE binding reactivity is significantly reduced with Bra r 1 mutants.

Furthermore, we also demonstrated that mutations in domain 2 were more effective in reducing IgE reactivity than changes in domain 1 (Fig. 5). This also seems to be true for other members of the calcium-binding pollen allergen class. Mutagenesis analysis of Bet v 4, the Bra r 1 homologue from birch, for instance, revealed that most Bet v 4-specific IgE antibodies were directed to the second calcium-binding domain. In case of Cyn d 7, the homologous allergen from Bermuda grass, a synthetic peptide of EF-hand domain 2 completely inhibited IgE binding to recombinant Cyn d 7, whereas a peptide of domain 1 could not lead to a decrease in IgE reactivity [8].

Treatment of allergic diseases is performed as allergen-specific immunotherapy, where increasing doses of allergens are injected for successful immunotherapy. However, the risk of anaphylactic reaction increases as well. To solve this problem, low IgE-binding forms of allergens would represent useful tools, since they have a reduced risk of such adverse reactions. Recently, hypoallergenic forms of allergens of mite Der f 2 and birch pollen Bet v 1 engineered by site-directed mutagenesis displayed reduced IgE-binding activity but still retained T cell epitopes essential for immunotherapy [14,26].

Recently, vaccination of mice with a multi-epitopic recombinant allergen of grass pollen suppressed an IgE antibody response but showed an increase in specific IgG in a dose-dependent manner [27]. After preincubation of Bet v 1 with a mouse monoclonal antibody, directed against the allergen, histamine release could be inhibited [28]. Furthermore, preincubation with human monoclonal antibodies against Bet v I inhibited IgE binding as well as histamine release [29]. It can thus be expected in allergen-specific immunotherapy that production of allergen-specific IgG antibodies might lead to a modulation in the immune response to allergens.

In conclusion, we engineered Ca^{2+}-binding deficient mutants of Bra r 1 which showed lower IgE reactivity than the wild-type protein. These recombinant mutant forms of Bra r 1 were injected into mice as a model system, to examine their potential for productive immune response. Specific IgGs against Bra r 1 mutants were produced and these IgGs could recognize native Bra r 1 in pollen extracts as well as rBra r 1 (Fig. 6). These results strongly suggest that recombinant Bra r 1 mutants with reduced anaphylactic index could be useful tools for allergen-specific immunotherapy. Furthermore, due to significant IgE cross-reactivity of Bra r 1 with other Ca^{2+}-binding pollen allergens, hypoallergenic variants of Bra r 1 have also the potential to be used as 'cross-protective' therapeutic agents for effective immunotherapy against allergies to related proteins such as Cyn d 7 and Bet v 4.

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