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Mutational epitope analysis and cross-reactivity of two isoforms of Api g 1, the major celery allergen

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Abstract

For better understanding the cross-reactivity between the major birch pollen and celery allergens, Bet v 1 and Api g 1, respectively, putative epitope areas and structurally important positions for IgE-binding of the isoforms Api g 1.01 and Api g 1.02 were point mutated. The IgE binding capacities were measured in ELISA, the IgE cross-reactivity between the isoforms, mutants and Bet v 1 investigated by ELISA-inhibition experiments with serum pools from patients with confirmed celery allergy (DBPCFC). Api g 1.01 displayed a clearly higher frequency and capacity of IgE binding than Api g 1.02. In Api g 1.01, substitution of lysine against glutamic acid at amino acid position 44, a key residue of the Bet v 1 "P-loop", increased the IgE-binding properties. Structural instability due to proline insertion at position 111/112 resulted in loss of IgE binding of Api g 1.01, but not of Api g 1.02. Between Api g 1.01 and Api g 1.02 only partial cross-reactivity was seen. The data suggest that the IgE epitopes of the two isoforms are distinct and that in contrast to Api g 1.01, the "P-loop" region plays an important role for IgE binding of celery allergic subjects to Api g 1.02. Understanding and investigation of the molecular mechanisms in celery allergy is an important step to generate hypoallergenic proteins for safe and efficacious immunotherapy of food allergy.

Keywords: Celery; Epitopes; Food allergy; Isoform; Mutation

1. Introduction

Allergy to celery tuber (celeriac) is a common plant food allergy in Europe, in particular in Switzerland, France and Germany. In Switzerland, it ranks among the most prevalent food allergies (Etesamifar and Wüthrich, 1998) and has been reported as the most frequent cause of food anaphylaxis (Rohrer et al., 1998). Approximately 50% of celery allergic subjects in Switzerland present a case history of systemic allergic reactions (Ballmer-Weber et al., 2000; Luttkopf et al., 2000). Clinically, celery allergy is associated to both birch pollen and mugwort pollen allergy, which is due to the existence of cross-reacting allergens in the respective pollen, which are also thought to represent the sensitizing allergens. The known allergens of celery tuber are the major allergen Api g 1.01 (Breiteneder et al., 1995) and its isoform Api g 1.02 (Hoffmann-Sommergruber et al., 2000), the minor allergen profilin Api g 4 (Scheurer et al., 2000) and Api g 5 (Bublin et al., 2003) a protein showing homology to FAD (flavin adenine dinucleotide) containing oxidases. The allergenicity of Api g 5 depends on *N*-glycans containing xylose and fucose residues and does not depend on the protein sequence. Several studies have shown that Api g 1 is the most important allergen in celery tuber (Breiteneder et al., 1995; Vieths et al., 1995; Hoffmann-Sommergruber et al., 1999; Luttkopf et al., 2000). The clinical presentation of celery allergy differs from other pollen related food allergies and appears to include more severe symptoms than oral allergy syndrome (Mari et al., 2005). Moreover, clinical reactivity to heat-processed celery has been confirmed by double-blind, placebo-controlled food challenges (Ballmer-Weber et al., 2002). Therefore, studying the IgE epitope structure of Api g 1 and IgE binding to its isoforms is of

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particular interest, because a specific set of epitopes or different stability characteristics could be responsible for a different clinical reactivity to this Bet v 1 related allergen. Due to the polyclonal nature of the IgE response, it is difficult to analyse directly the structure of immune complexes between IgE and allergens. Therefore, most studies on IgE epitopes of Bet v 1 related allergens have focused on site-directed mutagenesis (Ferreira et al., 1998; Scheurer et al., 1999; Neudecker et al., 2003; Wiche et al., 2005; Ma et al., 2006) or, more recently on direct affinity selection and enrichment of IgE binding surface structures utilising libraries of peptide mimics (Mittag et al., 2006). In the present study we focused on the "P-loop" which has been suggested as a major epitope area on Bet v 1 (Mirza et al., 2000) and homologous food allergens (Holm et al., 2001; Neudecker et al., 2003; Mittag et al., 2006). In addition, we found that introduction of a proline residue in position 112 of Bet v 1, or equivalent position of homologous food allergens led to an almost complete reduction of IgE binding capacity of Bet v 1 and the apple allergen Mal d 1 (Son et al., 1999) as well as the cherry allergen Pru av 1 (Scheurer et al., 1999; Neudecker et al., 2003) which could in the case of Pru av 1 be attributed to a complete loss of secondary structure. Therefore, the relevance of this position for the allergenicity of Api g 1 was also studied. In an initial screening the relevance of Api g 1.01 and Api g 1.02 was analysed for the first time in a study population in which food allergy to celery had been confirmed by double-blind, placebo-controlled food challenges. Our data revealed that the epitope structure of Api g 1 and its cross-reactivity of Bet v 1 with Api g 1 isoforms is complex and that mutation of positions equivalent to amino acid 112 of Bet v 1 has a significant impact on the structural stability of Api g 1 isoforms.

2. Materials and methods

2.1. Patients sera

In total 66 sera were included in this study. Twenty-three were from patients with a confirmed food allergy to celery tuber (celeriac) as demonstrated by a positive reaction in a double-blind, placebo-controlled food challenge (DBPCFC) with celery. Detailed clinical data such as case history of adverse reactions to celery and symptoms in response to DBPCFC as well as skin tests and serological data have been published elsewhere (Ballmer-Weber et al., 2000, 2002; Luttkopf et al., 2000). As controls, 22 sera were taken from birch pollen allergic patients without celery allergy, confirmed by a negative open food challenge test with fresh celery tuber, and 21 sera from healthy donors without allergy were included. Written informed consent was obtained from all study participants.

2.2. Cloning and mutagenesis of the recombinant celery allergens

Recombinant (r) Api g 1.0101 was generated as previously described (Neudecker et al., 2003). Api g 1.0201 cDNA (GenBank accession no. Z75662) was amplified from mRNA isolated from celery tuber by RT-PCR using the gene specific primers Api g 1.02(+)Nde 5′-GTA CAT ATG GGT GTC CAA AAG ACC GTG GTT GAG GCT and Api g 1.02(−)Bam 5′-CTA GGA TCC TCA AGC AAG AAA CTG CAA GTT TGC TAG (restriction sites underlined) and cloned in pCRII-Topo vector for sequence verification (Invitrogen, Karlsruhe, Germany). For expression the cDNA was directionally ligated in the pET-11a vector (Novagen, Schwalbach, Germany) between *Nde*I and *Bam*HI restriction sites. Mutagenesis reactions were performed with the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) using either the Api g 1.0101-pET11a or the Api g 1.0201-pET11a construct as template. Two Api g 1.0101 variants were generated. One single point mutation was inserted at position 44 ($K^{44}E$) using the mutagenic primers Apig1.01K^{44}E(+) 5′-GCT TAC AAG AGT GTA GAA ATC ***GAG*** GGA GAT GGT GGA CC-3′, Apig1.01K^{44}E(−) 5′-CCA GGT CCA CCA TCT CC***C TC***G ATT TCG ACA CTC TTG-3′ and one at amino acid position 111 ($S^{111}P$) primed by Apig1.01S^{111}P(+) 5′-CT GCT GAT GGA GGA ***CCC*** ATT TGC AAG ACC ACT GCC ATC-3′ and Apig101S^{111}P(−) 5′-GGC AGT GGT CTT CCA AAT ***GGG*** TCC TCC ATC AGC AGT TGG C-3′. One mutation of Api g 1.0201 at position 11($C^{111}P$) was introduced using the primers Apig102C^{111}P(+) 5′-GTG CCA ACC GAC GGA GGT ***CCC*** ATA GTG AAG AAC ACC ACC-3′ and Apig102C^{111}P(−) 5′-GGT GTT CTT CAC TAT ***GGG*** ACC TCC GTC GGT TGG CAC AAC AAC G3′ (mutated regions in bold italic). All mutation reactions were performed according to the manufacturers instructions.

2.3. Expression and purification of recombinant celery allergens

For expression as non-fusion proteins, the mutated plasmids were transformed into *Escherichia coli* BL21(DE3) competent cells (Novagen, Schwalbach, Germany). Protein synthesis was induced overnight at 23 °C by adding isopropyl-β-D-thiogalactoside (IPTG) (Carl Roth, Karlsruhe, Germany) to a final concentration of 1 mM, after cell growth was photometrically monitored to reach an optical density of 0.8 at 600 nm wavelength. Subsequently, cells were harvested by centrifugation, resuspended in lysis-buffer (50 mM NaH_2PO_4, 500 mM NaCl pH 7.0, 5 U/ml Benzonase (Merck, Darmstadt, Germany)) and lysed by repeated freezing and thawing. After 1 h rotation the extract was clarified by centrifugation for 30 min at 15,000 × *g*. The allergens were purified by preparative SDS-PAGE (Prep Cell Model 491, Bio-Rad, München, Germany) according to manufacturers instructions. The fractions that contained the pure allergen were pooled, dialyzed against 1/10 MOPS-buffer (2 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), 0.5 mM sodium acetate, 0.1 mM EDTA, pH 7.0), precipitated with acetone and redissolved in 1/10 MOPS-buffer. Recombinant non-fusion Api g 1.0101 was purified as described (Neudecker et al., 2003). Due to low expression levels obtained with the pET11a construct generated in the present study, rApi g 1.0201, was applied as C-terminally hexahistidine tagged pro-

tein (kindly provided by Dr. Jonas Lidholm, Phadia, Uppsala, Sweden). For detecting the purity of the recombinant allergens, 2 μg of each recombinant allergen were separated by SDS-PAGE according to the method of Laemmli (Laemmli, 1970) under reducing conditions in a Mini-Protean III cell (Bio-Rad, München, Germany) and analysed by Coomassie Brilliant Blue staining. Since in part different purification procedures were used for wild-type allergens and mutants, and since the wild-type, but not the mutant Api g 1.02 expression construct contained a histidine tag, experiments were performed to exclude that these differences would affect the results of IgE binding studies and potentially lead to misinterpretation of epitope data. To investigate potential negative effects on IgE reactivity of the purification procedure, used for the mutant proteins which contained a denaturing treatment with SDS, IgE binding to wild-type Api g 1.0101 purified according to the protocol described above was compared to the same allergen purchased from Biomay (Vienna, Austria) and to the material purified according to Neudecker et al. (2003), revealing identical IgE reactivity in ELISA. Similarly, non-fusion Api g 1.0201 purified by preparative SDS-PAGE was compared to the his-tagged material purified by immobilised metal affinity chromatography, and there was no reduction of IgE binding capacity found to be caused by the histidine tag. Further evidence for correct folding of the wild-type celery allergens resulted from the CD spectroscopic analysis performed in the present study.

2.4. Determination of specific IgE by ELISA

Measurement of the IgE binding capacity to the recombinant allergens was done by ELISA. Maxisorp™ plates (96 wells) (Nunc, Wiesbaden, Germany) were coated overnight at 4 °C with 50 ng protein per well in 100 μl PBS, thereafter blocked for 1 h with 200 μl/well PBS containing 2% BSA. Subsequently, duplicate wells were incubated overnight with 100 μl of serum diluted 1:4 in incubation buffer (PBS containing 0.05% Tween 20 and 0.1% BSA) (PBST). Bound specific human IgE was detected by 1 h incubation with 100 μl/well affinity-purified biotinylated anti-human IgE from goat, diluted 1:2000 (KPL, Gaithersburg, USA distributed by Medac, Wedel, Germany). Following 1 h incubation of 100 μl/well NeutrAvidin-horseradish peroxidase conjugate diluted 1:10,000 (Pierce, Rockford, USA), one hundred microliters per well 3,3′,5,5′-tetramethylbenzidine (TMB) was added as substrate. The enzymatic colour reaction was stopped after 20–30 min by adding 50 μl/well 3 M sulphuric acid. The optical densities (OD) were measured bichromatically at 450/630 nm in a SpectraMax 340 microplate reader (Molecular Devices, Ismaning, Germany). Apart from plate coating with celery allergens and mutants, all incubations were performed at ambient temperature. Between incubations, plates were washed four times with 300 μl/well PBST 0.05% and emptied. Results were evaluated as positive, if the OD of a serum sample was greater than three times the OD of a single control serum from a non-allergic subject, analysed in parallel on each individual microplate.

2.5. IgE ELISA-inhibition

For IgE ELISA inhibition experiments, the coating, blocking and detection of microwell plates was performed as described for the IgE ELISA. Pooled serum samples were used. Inclusion criteria for sera into pools are described in the results section. Optimal dilution of the single sera used in the serum pool was determined by analysing dilution series in the IgE ELISA. Sera were diluted to reach an OD between 0.8 and 1.0 to assure comparable levels of allergen-specific IgE from individual donors prior to serum pooling. The following inhibitors were applied: Api g 1.0101 wt (wild-type), Api g 1.01 $K^{44}E$, Api g 1.01 $S^{111}P$, Api g 1.0201 wt, Api g 1.02 $C^{111}P$ and Bet v 1a (Biomay, Vienna, Austria) at concentrations from 0.0001 μg/ml to 100 μg/ml. Fifty microliters of serum and 50 μl of inhibitors were incubated simultaneously in protein coated and blocked microwell plates over night. The residual IgE binding to microwells was measured as described above and the percentage of inhibition was calculated as follows (B: serum with inhibitor; BØ: serum without inhibitor; NSB: non-specific binding of immunoreagents):

$$\text{Inhibition}\,(\%) = \frac{\text{OD(B}\emptyset) - \text{OD(B)}}{\text{OD(B}\emptyset) - \text{OD(NSB)}} \times 100$$

2.6. CD spectroscopy

Circular dichroism (CD) studies of the recombinant allergens were performed on a Jasco J-810 spectropolarometer (Jasco, Groß-Umstadt, Germany) equipped with a CDF-426S Peltier temperature control system, interfaced with a Julabo F200 water bath (Julabo Labortechnik GmbH, Seelbach, Germany). CD-spectra were recorded at 20 °C using a step width of 0.2 nm and a bandwidth of 1 nm. The spectral range was 185–250 nm at 50 nm/min. To increase the signal-to-noise ratio ten scans were accumulated. Thermal denaturation was monitored by recording the ellipticity at a fixed wavelength of 222 nm while heating or cooling at 2 °C/min. Measurements were performed in a 1 mm path length quartz cuvette (200 μl, Hellma, Müllheim, Germany) at protein molarities between 4.8 μM and 7.7 μM in 10 mM potassium phosphate buffer (pH 7.0). The mean residue ellipticity ($[\theta]_{\text{m.r.w.}}$) was calculated from the measured ellipticity $[\theta]$ according to:

$$[\theta]_{\text{m.r.w.}} = 10 \times \frac{\theta}{cdN}$$

where θ denotes the measured ellipticity in mdeg, c the protein concentration in mM, d the light path in cm and N is the number of amino acid residues.

3. Results

3.1. Rationale of the study

The average amino acid sequence identity between the two Api g 1 isoforms and Bet v 1 is only about 40%. In addition, the two isoforms share only 50% sequence identity at the amino acid

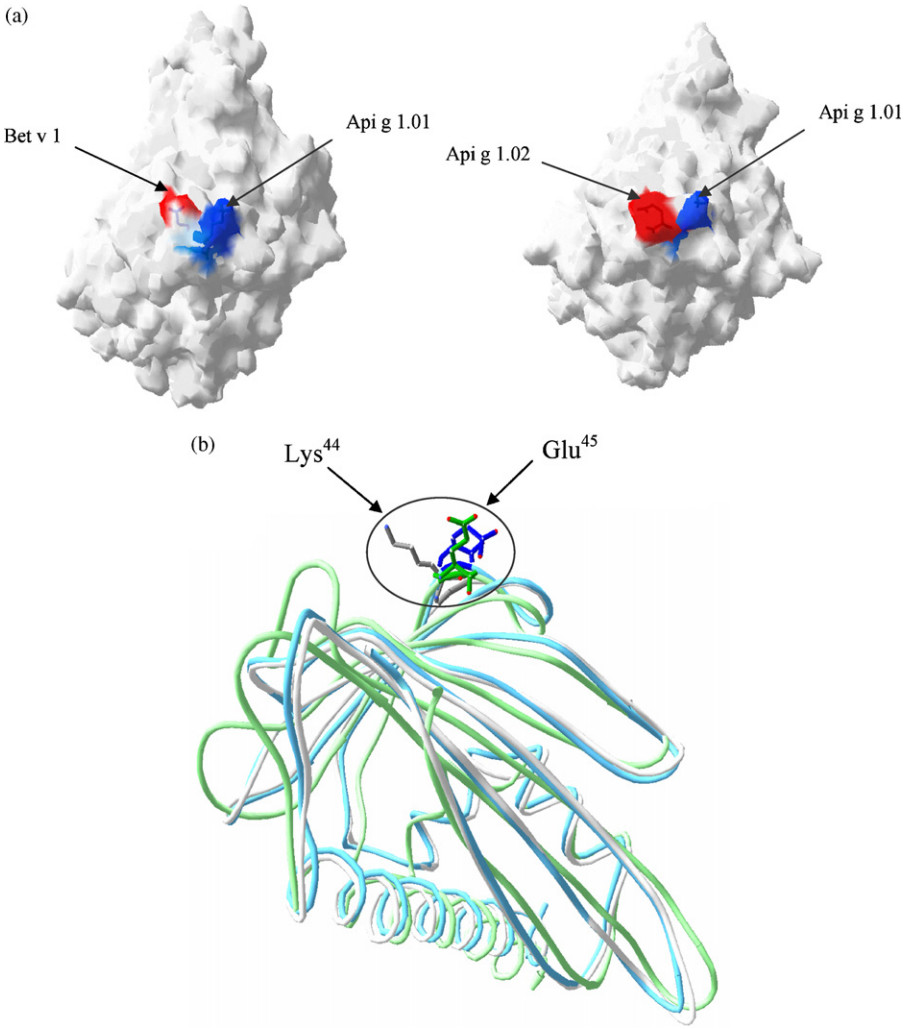

Fig. 1. Overlay of the structures of Bet v 1 (pdb: 1BTV), Api g 1.01 (pdb: 2BK0) and Api g 1.02. (a) Left: surface of the structures with charges at AA region 44/45 (red: negative charge, Bet v 1; blue, positive charge, Api g 1.01); right: surface of the structures with charges at AA region 44/45 (red: negative charge, Api g 1.02; blue, positive charge, Api g 1.01); (b) shows an overlay of the ribbon structure of the three allergens referenced to the orientation of AA region 44/45 (blue, Bet v 1; grey, Api g 1.01; green, Api g 1.02). Api g 1.02 was modelled by Swiss-Model using Api g 1.02 (pdb: 2BK0) as template.

level, whereas the P-loop region (amino acid position 44–53) is highly conserved in Bet v 1 homologous allergens (Neudecker et al., 2003). Interestingly, Api g 1.01 differs from Api g 1.02 and Bet v 1 in this region. The latter two carry a negatively charged glutamic acid (Glu^{45}) at amino acid position 45, whereas Api g 1.01 carries a positively charged lysine (Lys^{44}) at the corresponding position (Fig. 1a). Sterical differences at AA position 44 (Api g 1.01) and amino acid position 45 for Bet v 1 and Api g 1.02 are shown in Fig. 1b. Consequently, we mutated Api g 1.01 in direction towards Bet v 1 with special regard to amino acid position 45. For investigation of the impact of preserved protein structure on total IgE binding, the amino acid at position 111, corresponding to amino acid position 112 at Bet v 1, was replaced by proline in both isoforms, expecting that this mutation would in the same way lead to unfolded proteins as shown for Pru av 1, the major cherry allergen (Neudecker et al., 2003). To take into account that different clinical profiles might be the results of differences in epitope recognition, IgE reactivity of sera from subjects with confirmed celery allergy was compared to IgE binding of birch allergic subjects without celery allergy.

3.2. *Purification, folding and stability of the recombinant proteins*

Purification of the mutated recombinant proteins by continuous elution preparative SDS-PAGE resulted in proteins of high purity (>95%) as assessed by SDS-PAGE and Coomassie brilliant blue staining (Fig. 2). CD spectroscopy revealed that all recombinant proteins apart from Api g 1.02 $C^{111}P$ had secondary structures typical for members of the Bet v 1 allergen family (Fig. 3). A difference between the maxima of Api g 1.01 and Api g 1.02 proteins was found that corresponded to findings with the homologous allergen isoforms from carrot (Ballmer-Weber et al., 2005) and suggested different portions of secondary structure elements for the two wild-type isoforms. As expected, the mutant Api g 1.02 $C^{111}P$ exhibited the CD spectrum of a random coil protein. Surprisingly, the CD spectrum of the mutant Api g 1.01

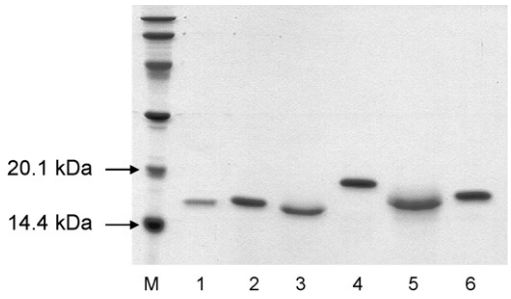

Fig. 2. Analysis of purified recombinant allergens by electrophoresis 2 μg protein per slot, separated on SDS-PAGE (15% acrylamide, reducing conditions); lane 1, Api g 1.01; lane 2, Api g 1.01 $K^{44}E$; lane 3, Api g 1.01 $S^{111}P$; lane 4, Api g 1.02; lane 5, Api g 1.02 $C^{111}P$; lane 6, Bet v 1a (Biomay); M, molecular-mass marker (LMW, GE Healthcare, Uppsala, Sweden).

$S^{111}P$ indicated that the protein contained elements of secondary structure, and comparison with the spectrum of the wild-type allergen suggested that the protein was partially folded. To verify that the point mutation introduced into Api g 1.01 $S^{111}P$ led to a protein with modified structural properties and stability characteristics, a melting curve analysis was performed by CD spectroscopy (Fig. 4). The wild-type isoform Api g 1.01 unfolded at a transition temperature around 70 °C, whereas Api g 1.01 $S^{111}P$ already showed unfolding at a transition temperature between 40 °C and 50 °C. Therefore, the structure of the proline mutant had a clearly lower thermal stability than the wild-type isoform. The mutated isoform Api g 1.01 $K^{44}E$ displayed almost the same melting curve as the wild-type isoform Api g 1.01, with a transition temperature slightly lower than 70 °C. Isoform Api g 1.02 unfolded at a transition temperature of 55 °C (data not shown) and was therefore found to be less stable than the isoform Api g 1.01.

3.3. IgE binding capacity of isoforms and mutants

The IgE binding capacity of all recombinant proteins was determined by IgE ELISA. All OD's were reduced

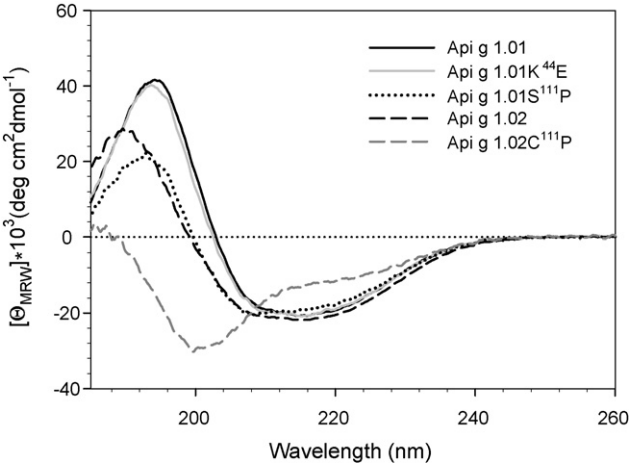

Fig. 3. Circular dichroism (CD) spectra of Api g1 isoforms and mutants.

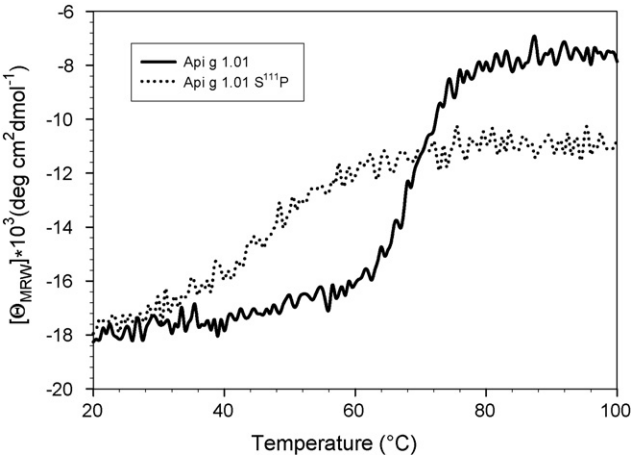

Fig. 4. Denaturation curves of Api g 1.01 and Api g 1.01 $S^{111}P$. Transitions were monitored by the CD signal at 222 nm.

by the background, measured by a non-specific non-allergic control.

Seventy-four percent (16/23) of the celery allergic patients reacted to Api g 1.01 (mean OD 1.229, OD 0.02–3.57) and 48% (11/23) to Api g 1.02 (mean OD 0.233, OD 0.02–2.7). Not only was Api g 1.02 recognised by IgE from a lower number of celery allergic subjects, also its IgE binding capacity was significantly lower in comparison to Api g 1.01 (Fig. 5a) and correlated with a lower IgE binding capacity concluded from qualitative Western blotting data (Hoffmann-Sommergruber et al., 2000). A similar difference between the isoforms was seen in the birch pollen allergic patients without celery allergy. Due to clinically insignificant cross-reactivity, 41% (9/22) reacted to Api g 1.01, but only 14% (3/22) to Api g 1.02 (data not shown). When comparing the IgE binding capacity of the unmutated isoform Api g 1.01 to that of the Bet v 1-approximated mutant Api g 1.01 $K^{44}E$, the mutated form seemed to exhibit a slightly stronger IgE binding capacity for the majority of sera from celery allergic subjects (Fig. 5b). The Api g 1.01 $S^{111}P$ mutant with a destabilized structure showed a significantly reduced IgE binding capacity to the sera from celery allergic patients in comparison to the wild-type allergen. Fifty-two percent (12/23) of the patients sera still had IgE binding to this mutant, however with a clearly reduced intensity (mean OD 0.335, OD 0.01–2.7) (Fig. 5c). The loss of secondary structure of the isoform Api g 1.02 (mean OD 0.233, OD 0.02–2.7) after insertion of proline at amino acid position 111, did not lead to a decreased IgE binding capacity (mean OD 0.306, OD 0.11–3.23) compared to the wild-type isoform Api g 1.02 (47.8% versus 30.4% of sera) (Fig. 6). Comparable IgE-binding pattern to the Api g 1 isoforms and mutants were observed with the sera from birch pollen allergic patients. However, the overall IgE binding capacity was clearly lower than in sera from patients with confirmed celery allergy (data not shown). The non-allergic control sera showed no detectable IgE-binding to the isoforms, nor to the mutants of Api g 1. The measured OD of all negative sera were lower than three times the OD of the control serum.

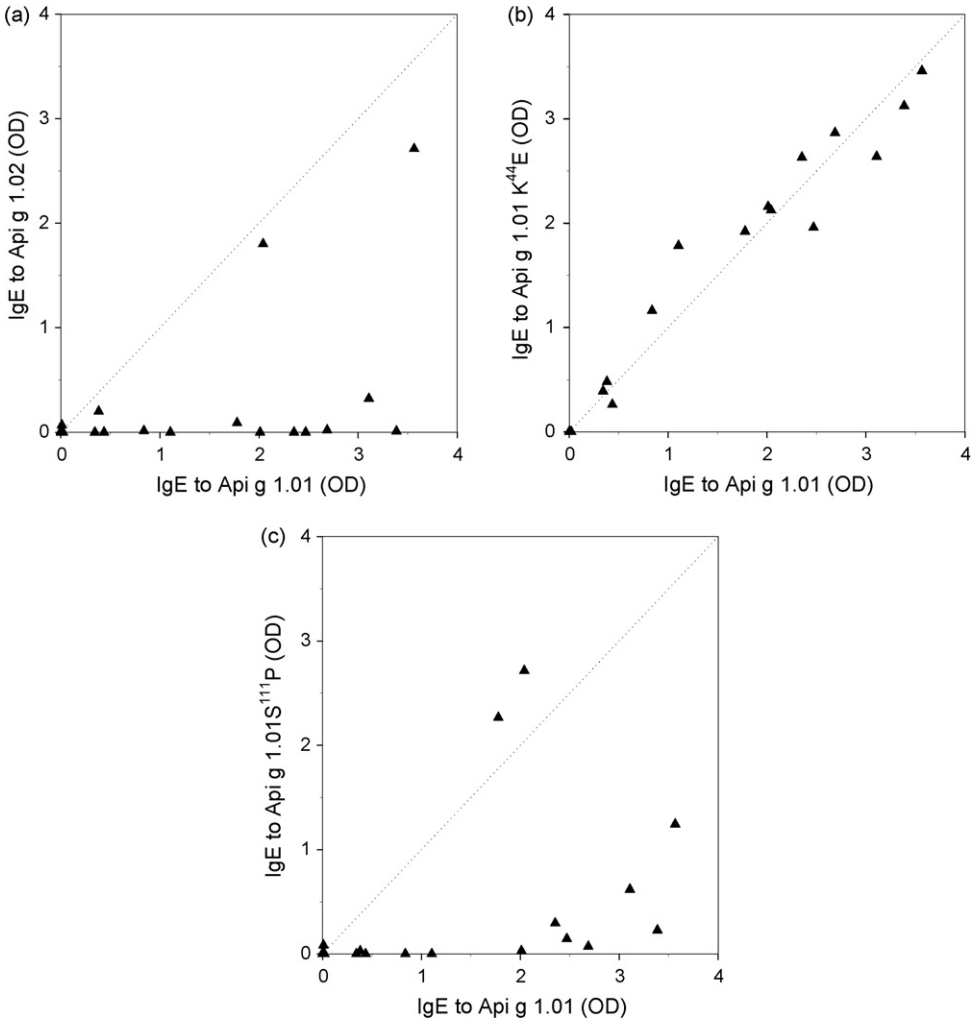

Fig. 5. Comparison of the IgE-binding capacity of Api g 1 Isoforms and their mutants, assessed by means of ELISA with sera of celery allergic patients. (a) 1.01 vs. 1.02; (b) 1.01 vs. 1.01$K^{44}E$; (c) 1.01 vs. 1.01$S^{111}P$.

3.4. IgE cross-reactivity studies indicate different epitopes responsible for IgE binding to Api g 1.01 and Api g 1.02

ELISA-inhibition experiments were performed for quantitative investigation of the impact of mutation of the Api g 1 isoforms on the IgE binding capacity. Different solid phase allergens and two different serum pools from celery allergic subjects presenting different sensitization profiles were used. One serum pool consisted of four sera with sensitization to Api g 1.01 only (pool A), the other pool contained three sera with IgE to both Api g 1.01 and Api g 1.02 (pool B). Monosensitization to Api g 1.02 was not seen in any of the sera. Microplates were coated with either Api g 1.01 or Api g 1.02 and the inhibition of IgE binding was determined by simultaneous incubation of serum pools with Api g 1 isoforms or mutants in allergen coated wells (Fig. 7).

In case of Api g 1.01 as solid-phase allergen, Bet v 1 displayed the highest inhibition potency, in both pool A and pool B (Fig. 7a and b). Bet v 1 had between two and three orders of magnitude higher potency of inhibition than the isoform Api g 1.01, when used as self-inhibitor (Fig. 7a and b). The IgE binding capacity of the Bet v 1-like mutant Api g 1.01 $K^{44}E$ was higher than that of the wild-type isoform Api g 1.01. The difference between the two allergens was greater than would be expected by chance and a statistical significance was found by paired *t*-test ($p = 0.034$). The Api g 1.01 $S^{111}P$ mutant with destabilized structure showed no inhibition of IgE binding in serum pool A. Similarly, Api g 1.02 did not inhibit IgE binding to Api g 1.01 in sera from subjects mono-sensitized to Api g 1.01 (Fig. 7a). Using pool B with sensitization to both isoforms in inhibition experiments with Api g 1.01 coated plates, inhibition of IgE-reactivity by Bet v 1, Api g 1.01 $K^{44}E$ and Api g 1.01 was comparable to that of pool A. The difference between Api g 1.01 and Api g 1.01$K^{44}E$ in this case is also statistically significant (paired *t*-test; $p = 0.017$). Moreover, Api g 1.02 inhibited IgE-binding to Api g 1.01 up to 40%: However, this effect was only observed when the inhibitor was applied at approximately 10–100-fold higher concentration (Fig. 7b). Api g 1.01 $S^{111}P$ displayed only little inhibition of up to 20% at high concentrations. Api g 1.02$C^{111}P$ with destroyed structure also showed inhibition up to 40% (Fig. 7b). With Api g 1.02 on the solid phase and serum pool B, Api g 1.02 self-inhibition showed the highest inhibition-potency

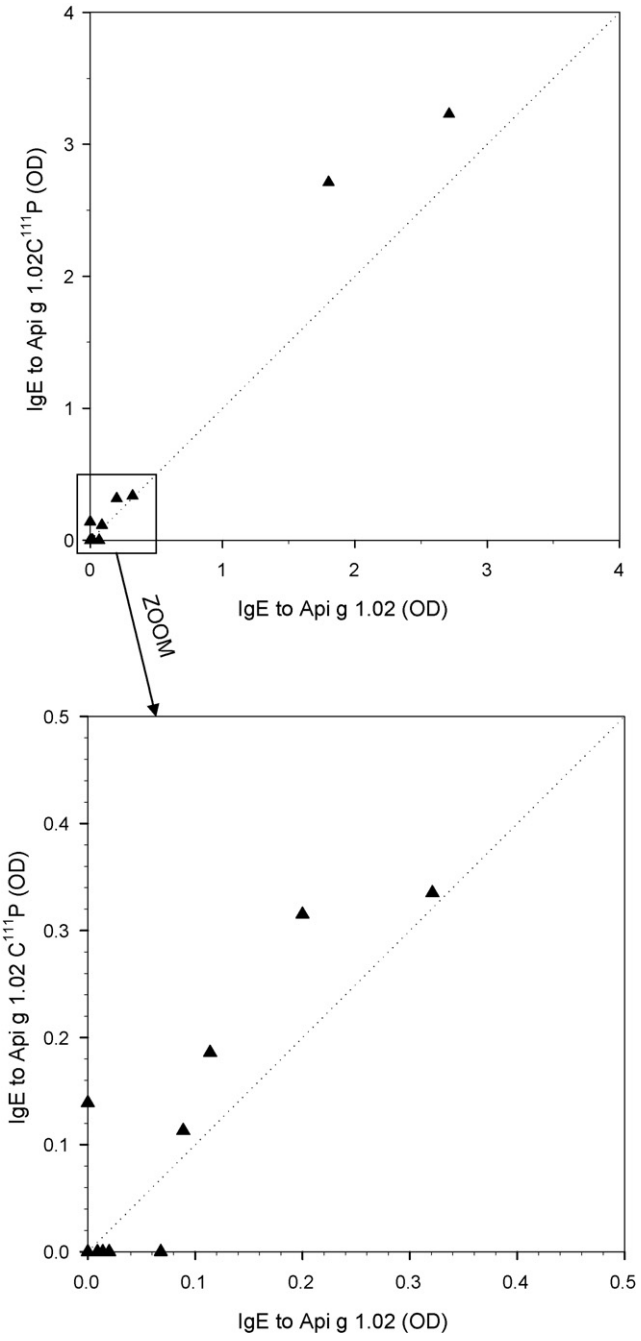

Fig. 6. Comparison of the IgE-binding capacity of Api g 1.02 and Api g 1.02 $C^{111}P$, assessed by means of ELISA with sera of celery allergic patients.

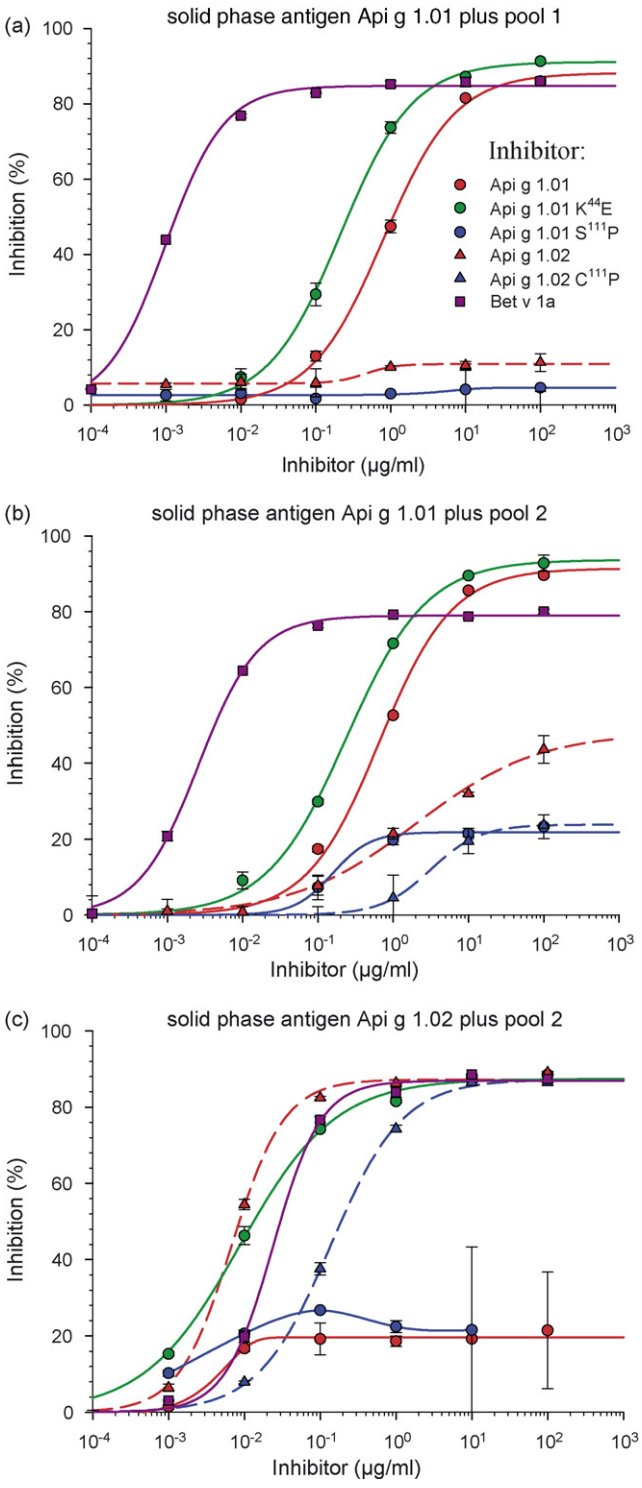

Fig. 7. Inhibition of IgE binding to Api g 1.01 (a, b) and Api g 1.02 (c) with different pool sera. (a) Pool 1, patients sensitized only to Api g 1.01, (b, c) pool 2, patients sensitized both to Api g 1.01 and Api g 1.02.

(Fig. 7c). A similar inhibition potency was observed in case of the Bet v 1-like mutant Api g 1.01 $K^{44}E$, whereas the unmutated Api g 1.01 indicated no relevant inhibition (maximum 20%). Incubation of serum pool B with Bet v 1 led to complete inhibition, but the concentration had to be approximately four-fold higher than with Api g 1.02. The Api g 1.02 $C^{111}P$ mutant with destroyed secondary structure showed complete inhibition at high concentrations, whereas the proline mutant of Api g 1.01 (Api g 1.01 $S^{111}P$) led to an inhibition of only 20% (Fig. 7c).

4. Discussion

Structures that bind IgE are relevant for clinical reactivity and changes in epitope structures may lead to drastically different IgE binding. Therefore we wanted to investigate the influence of directed mutation of Api g 1 isoforms on their IgE binding

capacity and analysed their cross-reactivity to the homologous allergen from birch pollen, Bet v 1.

Up to now only one comparative study on the IgE binding capacity of Api g 1 isoforms has been performed. This study analysed sera from patients with a positive case history of celery allergy by IgE Western blotting and provides only limited information on the IgE binding capacity due to the qualitative nature of the method (Hoffmann-Sommergruber et al., 2000). In contrast, in our study, sera from subjects whose food allergy had been confirmed by DBPCFC (Ballmer-Weber et al., 2000), which represents the only scientifically accepted test for food allergy (Bindslev and Poulsen, 1998; Bindslev-Jensen et al., 2004), were analysed by ELISA in order to provide refined clinical information as well as a quantitative readout for IgE binding capacity. The results confirm that Api g 1.01 is the more relevant isoform of the celery allergen not only in regard to the frequency of sensitization, but also in terms of IgE binding capacity.

Low sequence identity of the two isoforms Api g 1.01 and Api g 1.02 among each other and also with Bet v 1 (Hoffmann-Sommergruber et al., 2000) prompted us to investigate the influence of replacing amino acids directly involved in the epitope structure and also affect the folding of these isoforms by introducing directed mutations.

Therefore we produced one mutant, Api g 1.01 $K^{44}E$, in which a key residue of the P-loop was modified in the direction of Bet v 1 (Mirza et al., 2000; Neudecker et al., 2001) and mutants with supposedly destabilized structure, Api g 1.01 $S^{111}P$ and Api g 1.02 $C^{111}P$, and compared their IgE binding capacity to that of the wt mutants and Bet v 1. The IgE binding experiments were done with sera from celery allergic patients, birch pollen allergic patients without celery allergy and a panel of non-allergic subjects. We distinguished between patients that showed IgE only against Api g 1.01 and sera from patients with IgE against both Api g 1.01 and Api g 1.02.

The clearest difference in IgE binding capacity of the mutants was seen when a proline was introduced into Api g 1.01. Amino acid change $S^{111}P$ with Api g 1.01 led to an almost complete loss of IgE binding capacity, whereas the same exchange $C^{111}P$ in Api g 1.02 had no statistically significant effect on the IgE binding capacity. This result was confirmed by direct IgE measurements as well as with the IgE-inhibition ELISA (Figs. 5–7). Sera from patients mono-sensitized to Api g 1.01 showed no reaction to both mutants that contained an introduced proline at amino acid position 111 (Api g 1.01 $S^{111}P$ and Api g 1.02 $C^{111}P$) and there was no relevant inhibition detectable with the latter two antigens in an IgE-inhibition experiment using Api g 1.01 as solid phase protein (Fig. 7a). When sera from subjects sensitized to both Api g 1 isoforms were used, the proline mutant of Api g 1.02 showed partial inhibition of the IgE binding to Api g 1.01 as solid phase antigen (Fig. 7b). Similarly, the mutant Api g 1.02 $C^{111}P$ showed a complete inhibition with Api g 1.02 as solid phase protein. Self-inhibitor wt Api g 1.02 was a 20-fold more potent than the unfolded mutant (Fig. 7c). These observations have to be explained in the context of structural data obtained by CD spectroscopy: Although the proline mutant of Api g 1.01 showed Bet v 1-like secondary structure, thermal denaturation analysis indicated that the secondary structure was much more susceptible to denaturation compared to the unmutated protein. This structural instability likely resulted in the observed lack of IgE binding capacity in ELISA measurements and inhibition tests and confirmed the importance of folding for the epitope structure of Api g 1.01. The interpretation of results obtained with Api g 1.02 and its proline mutant appears to be more complex: CD spectroscopy revealed a complete loss of secondary structure, while IgE binding capacity and IgE inhibition potency remained largely unaffected. This observation may be explained by the existence of sequential epitopes on Api g 1.02. However, the possibility that the allergen preparation used contained a very low amount of correctly folded mutant protein or secondary structure formed transiently and undetectable by CD spectroscopy cannot be entirely excluded.

Birch pollen-related food allergy is thought to be caused by cross-reacting IgE epitopes on the homologous pathogenesis related proteins, found in many pollen related food. For the major allergen in birch pollen Bet v 1 (Mirza et al., 2000; Spangfort et al., 2003) as well as in food allergens, for example Pru av 1 from cherry (Scheurer et al., 1997, 1999; Neudecker et al., 2003; Wiche et al., 2005) the P-loop (AA 41–52) has been described as a major IgE binding region and Glu45 was described as being highly important for IgE binding (Mirza et al., 2000; Neudecker et al., 2003). In contrast to Api g 1.01, the isoform Api g 1.02 comprises a P-loop region homologous to Bet v 1 at amino acid position 45. Despite this homology the majority of sera show stronger IgE reactivity to Api g 1.01 while Api g 1.02 was identified as low IgE binder in our study. These data may be seen as evidence that the epitope structure of Api g 1 is different from Bet v 1. When the mutant Api g 1.01 $K^{44}E$ containing a P-loop mutated in the direction of Bet v 1 was tested in IgE ELISA and ELISA-inhibition experiments with Api g 1.01 as solid phase antigen, this mutant showed a higher binding capacity than the wild-type form of Api g 1.01 (Fig. 7a and b). This increased reactivity to Api g 1.01 $K^{44}E$ suggests that an epitope was formed that is relevant for birch pollen allergic subjects sensitized to Bet v 1, but not primarily for celery allergic subjects. This view is further supported by the fact that similar IgE binding data were obtained in birch pollen allergic subjects without celery allergy (data not shown).

However, when IgE-inhibition experiments were performed with Api g 1.02 as solid phase antigen (Fig. 7c), the Api g 1.01 mutant with a Bet v 1-like P-loop (Api g 1.01 $K^{44}E$), showed a high IgE binding capacity very similar to Api g 1.02, whereas the wild-type isoform of Api g 1.01 presented weak partial cross-reactivity with Api g 1.02 (maximum inhibition 20%). This led to the conclusion that in contrast to Api g 1.01, the P-loop region plays an important role for IgE binding of celery allergic subjects to Api g 1.02, and that the IgE epitopes of the two isoforms are distinct, which is further evidenced by the cross-inhibition experiments presented in Fig. 7a and b.

In this study we showed that in case of allergic reaction to celery, in particular the reaction to Api g 1.01, the described cross-reacting IgE binding P-loop region, is not a relevant epitope for IgE antibody binding as described for other pollen related major food allergens such as Pru av 1 from cherry. Because Bet v 1 is the known initiator of sensitization to Api g

1 (Bohle et al., 2003), there has to be one or more other relevant IgE epitopes on the allergen Api g 1.01 and also on Bet v 1. In case of the isoform Api g 1.02 the P-loop region seemed to play a role in IgE binding, but only few of the patients showed high IgE binding to this protein. Bohle et al. (2003) described the region around AA 109–126 as T-cell epitope and Wiche et al. (2005) described the region around AA 28 in Pru av 1 as a putative IgE binding epitope. In further investigations these regions could be included in epitope studies to identify the epitope(s) relevant for the clinical phenomenon of allergy to celery.

Because of a high risk of severe side effects, immunotherapy of food allergy is not yet possible. Studying the molecular basis of food protein allergenicity is an important step towards the development of mutated proteins derived from allergen sequences with modified structures resulting in destroyed B-cell epitopes, but intact T-cell epitopes (Bohle et al., 2003, 2005). While our P-loop mutants of Api g 1 demonstrate that the IgE epitope structure of Bet v 1 homologues is complex and not identical to Bet v 1, the proline mutants of the Api g 1 isoforms generated in the present study could be candidate molecules for such immunotherapy vaccines. Their structural stability is strongly decreased resulting in a diminished IgE binding capacity while the sequence is almost unchanged which likely results in the preservation of T-cell epitopes.

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