BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 197–204 (1997)
ARTICLE NO. RC976860

Molecular Characterization, Expression in *Escherichia coli,* and Epitope Analysis of a Two EF-Hand Calcium-Binding Birch Pollen Allergen, Bet v 4

Anna Twardosz,* Brigitte Hayek,* Susanne Seiberler,* Luca Vangelista,† Lena Elfman,‡ Hans Grönlund,‡ Dietrich Kraft,* and Rudolf Valenta*,[1]

**Institute of General and Experimental Pathology, AKH, University of Vienna, Austria; †European Molecular Biology Laboratory (EMBL), Heidelberg, Germany; ‡Pharmacia & Upjohn, Uppsala, Sweden*

Received May 15, 1997

Birch pollen belongs to the most potent elicitors of Type I allergic reactions in early spring. Using serum IgE from a birch pollen allergic patient, two cDNA clones (clone 6 and clone 13) were isolated from a birch pollen expression cDNA library constructed in phage λgt11. Clone 6 encoded a 9.3 kD two EF-hand calcium-binding protein, designated Bet v 4, with significant end to end sequence homology to EF-hand calcium-binding allergens from weed and grass pollen. Recombinant Bet v 4, expressed as β-galactosidase fusion protein, reacted with serum IgE from approximately 20% of pollen allergic individuals. Depletion of allergen-bound calcium by EGTA treatment lead to a substantial reduction of IgE-binding to Bet v 4, indicating that protein-bound calcium is necessary for the maintenance of IgE-epitopes. The greatly reduced IgE-binding capacity of clone 13, a Bet v 4 fragment that lacked the 16 N-terminal amino acids, indicated that the N-terminus contributes significantly to the proteins IgE-binding capacity. By IgE-inhibition experiments it was demonstrated that recombinant Bet v 4 shared IgE-epitopes with natural Bet v 4 and a homologous timothy grass pollen allergen. Recombinant Bet v 4 may therefore be considered as a relevant crossreactive plant allergen, which may be used for diagnosis and treatment of patients suffering from multivalent plant allergies.

Almost 20% of the population in industrialized countries mount IgE-antibody responses against otherwise harmless environmental antigens that are derived from airborn particles (pollen, mite feces, animal hair/dander, moulds) (1). Crosslinking of effector cell-bound IgE antibodies by allergens leads then to the release of biological mediators (e.g. histamine) which causes immediate type symptoms (allergic rhinitis, conjunctivitis, asthma, dermatitis, anaphylactic shock), the hallmark of Type I allergy. Pollen of white birch (*Betula verrucosa*) as well as of related trees belonging to the order *Fagales* represent the most important and potent allergen sources in early spring (2). The relevant allergens of these trees and plant-derived food contain crossreactive IgE epitopes and it has therefore been proposed that diagnosis and probably therapy of tree pollen allergy may be performed with selected allergens from only one source (3-5). In order to obtain recombinant tree pollen allergens for diagnostic and therapeutic purposes cDNAs coding for several tree pollen allergens have been isolated and expressed in *Escherichia coli* (6-11) The major birch pollen allergen, Bet v 1, represents a 17 kD allergen with significant sequence homology to pathogenesis-related plant proteins (6) and has been proposed to have RNAse properties (12). More than 95% of tree pollen allergic patients display IgE-reactivity against Bet v 1 and due to structural similarities with related plant food allergens frequently suffer from allergic food intolerance (5). Birch profilin, Bet v 2, represents the probably most cross-reactive allergen described so far (7, 13). Profilins are small (14 kD) eukaryotic actin-, PIP- and proline-rich peptide-binding proteins which share a surprisingly high structural similarity (14, 15). Profilins from different unrelated species are recognized by approximately 20% of all plant allergic individuals and therefore have been termed "panallergens" (13). More recently, cDNAs coding for Bet v 3 were isolated (11, 16). Bet v 3 is a three-EF hand calcium-binding birch pollen allergen which contained IgE-epitopes that were sensitive to calcium depletion.

Here we describe the isolation and molecular charac-

[1] To whom correspondence should be addressed.

0006-291X/97 $25.00

terization of a cDNA coding for another highly conserved calcium-binding birch pollen allergen, Bet v 4. Bet v 4 represents a two EF-hand calcium-binding allergen which is highly homologous to calcium-binding allergens from weeds (*Brassica napus*) (17), grasses (*Cynodon dactylon*) (18) and trees (*Olea europea*) (19).

MATERIALS AND METHODS

Biological materials, phage, E. coli strains, plasmids, and patients sera. Birch (*Betula verrucosa*) and timothy grass (*Phleum pratense*) pollen were purchased from Allergon (Välinge, Sweden). Eco R I cut dephosphorylated λgt11 phage DNA was obtained from Amersham (Buckinghamshire, UK). *Escherichia coli* strains Y1090 [*hsd* ($r_k^-m_k^+$) *lac* U169, *Pro* A^+, *Ion*-, *ara* D 139, *Str* A, *Sup* F *trp* C22:Tn10 (pMC9)] and Y1089 [*hsd* ($r_k^-m_k^+$) *lac* U169, *Pro* A^+, *Ion*-, *ara* D 139, *Str* A, *hfl* F 150 chr:Tn10 (pMC9)] were purchased from Amersham (Buckinghamshire, UK) and *E. coli* XL1-Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacl*^{q}*ZΔM15*Tn*10* (Tetr)] was from Stratagene (La Jolla, CA). Plasmid pUC18 was obtained from Boehringer (Mannheim, Germany) and plasmid pBSk+ was purchased from Stratagene (La Jolla, CA).

Birch, grass, olive, maize and mugwort allergic patients were characterized by positive case history and skin reactions. The presence of serum IgE antibodies with specificity for the pollen extracts was confirmed by RAST (radioallergosorbent test) analyses. The allergen profile recognized by sera from the birch pollen allergic individuals was further analyzed using nitrocellulose blotted birch pollen extract and recombinant birch pollen allergens, rBet v 1, rBet v 2 and rBet v 3 as described (20, 21).

Protein extracts. Pollen protein extracts were prepared by homogenizing 3 g pollen in 50 ml distilled water containing 5mM PMSF with an ultraturrax (IKA, Heidelberg, Germany) and shaking at 4°C for 1 hour. Extracts were then centrifuged at 20.000xg for 30′ at 4°C to remove insoluble particles (22). Supernatants were lyophilized, checked for quantity and quality of extracts by SDS-PAGE (23) and Coomassie Blue staining (24).

Recombinant β-galactosidase fusion allergens were obtained by lysis of lysogenic *E. coli* Y1089 in SDS-sample buffer and boiling for 5 minutes prior to SDS-PAGE.

IgE-immunoscreening of the birch pollen cDNA expression library. A birch pollen expression cDNA library was constructed in phage λgt11 as described previously (7). Approximately 20.000 pfu per plate were used to infect *E. coli* Y1090 and plated onto LB plates (145 mm diameter) containing 100mg/l ampicillin. In total approximately 250.000 pfu were screened. The synthesis of β-gal-fusion proteins was induced by overlay with nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) that had been soaked in 10 mM IPTG. IgE-binding clones were detected with serum IgE from a patient suffering from multivalent plant allergies using ^{125}I labeled anti-human IgE antibodies (RAST, Pharmacia, Uppsala, Sweden) as secondary antibody (7). Phage expressing IgE-binding birch pollen proteins were purified by two rounds of rescreening, amplified and stored at 4°C.

Selection of IgE-binding phage clones, subcloning into plasmids and DNA sequence analysis. Twenty-three IgE-binding clones were obtained by IgE-immunoscreening with serum IgE of a patient suffering from multivalent plant allergy. The testing of these clones with sera from birch pollen allergic individuals with well characterized IgE-reactivity profile to birch pollen allergens indicated that clones 6 and 13 represented novel allergens. Phage DNA from clone 6 and clone 13 phage was isolated using a plate lysate method (25). The DNA was cut with Kpn I/Sac I in order to obtain the allergen-encoding cDNA flanked on each side by approximately 1000 base-pairs of λgt11 DNA. The KpnI/SacI fragments of clone 6 and clone 13 were subcloned into plasmid pUC18 and pBSk+, respectively, and transformed in *E. coli* XL-1 Blue. Plasmid DNA was purified using Quiagen tips (Quiagen, Hilden, Germany) and both DNA strands were sequenced by the chain termination method (26) using λgt11 forward: 5′ CGG GAT CCC GGT TTC CAT ATG GGG ATT GGT GGC 3′, λgt11 reversed: 5′ CGC GGA TCC CGT TGA CAC CAG ACC AAC TGG TAA TG 3′ and sequence specific internal primers (MWG, Ebersberg, Germany), ^{35}S dCTP (NEN, Stevenage, UK) and a T7 sequencing kit (Pharmacia, Uppsala, Sweden).

DNA and deduced amino acid sequence analysis, structural predictions. The clone 6 and clone 13 cDNA sequence was analyzed with the McVector Program (Kodak, Rochester, USA). DNA and deduced amino acid sequences were compared with the NIH data bases using the program BlastN and BlastP. Multiple sequence alignment of Bet v 4 and homologous plant allergens was done with CLUSTAL W (27) and, if necessary, edited by hand. The GDE sequence editor (S. Smith, Harvard University, Cambridge, MA) and COLORMASK (J. Thompson, EMBL, Heidelberg, Germany) were used to color conserved residues with related properties (27). Protein secondary structure prediction was performed using the PHD program on the EMBL PredictProtein server (28, 29).

Expression of recombinant Bet v 4 in E. coli; IgE-binding capacity of recombinant Bet v 4 and a recombinant Bet v 4 fragment. Recombinant clone 6-β-gal fusion protein and, for control purposes, β-gal alone were produced after infection of *E. coli* Y1089. Lysogenic *E. coli* Y1089 were grown in LB containing 100mg/l ampicillin, 0.4% w/v maltose and 10mM $MgSO_4$ at 32°C to an OD_{600} of 0.4. The growth temperature was then rised to 43°C for 30′ and expression of recombinant proteins was induced by adding IPTG to 5mM final concentration and further growth at 37°C for 1 hour. *E. coli* cells were harvested by centrifugation and protein extracts were prepared by lysis of the pellets in SDS-sample buffer. According to analytical SDS-PAGE and Coomassie Blue staining, comparable amounts of protein extracts containing clone 6-β-gal or β-gal alone were separated by 10% preparative SDS-PAGE and transfered to nitrocellulose by electroblotting (30). Nitrocellulose strips were probed with serum IgE as described (13).

Calcium-dependent modulation of IgE-binding to recombinant Bet v 4. Nitrocellulose filters containing recombinant Bet v 4 (clone 6), a recombinant Bet v 4 fragment (clone 13) and for control purposes recombinant Bet v 1 (6), recombinant Bet v 2 (7) and *E. coli*-phage proteins were prepared by plaquelift as described for the immunoscreening (21). Filters were cut into equally sized sectors which were probed with serum IgE from Bet v 4-allergic patients, and, for control purposes, with sera from a Bet v 4-negative birch pollen allergic as well as from a non-allergic individual and buffer without addition of serum. During blocking, probing and washing one set of filters was treated with buffer A (50mM Naphosphate, pH 7.5, 0.5% w/v BSA, 0.5% v/v Tween 20, 0.05% NaN_3) containing 5mM EGTA and the other set of sectors was exposed to buffer A containing 5mM $CaCl_2$. Bound IgE-antibodies were detected with ^{125}I labeled anti-human IgE-antibodies (RAST, Pharmacia, Uppsala) visualized by autoradiography and quantified by γ-counting of the sectors in a γ-counter (Wallac, Turku, Finland).

IgE-inhibition experiments. IgE-epitopes shared by recombinant, natural Bet v 4 and Bet v 4 related allergens in timothy grass pollen were studied by IgE-inhibition experiments as described (31). One:10 in buffer A diluted sera were preadsorbed with equally sized filter sectors containing recombinant Bet v 4 (clone 6) and, for control purposes, *E. coli*-λgt11 phage proteins overnight at 4°C. The preadsorbed sera were then probed with nitrocellulose blotted birch pollen and timothy grass pollen extracts, respectively. Bound IgE-antibodies were detected as above and visualized by autoradiography.

```
                                    gaa ttc cca aca aca gtg gaa aca aaa

ATG GCT GAT GAT CAT CCA CAG GAC AAG GCT GAA CGC GAG CGC ATT   45
Met Ala Asp Asp His Pro Gln Asp Lys Ala Glu Arg Glu Arg Ile   15

TTC AAG CGC TTT GAC GCC AAT GGC GAT GGT AAA ATC TCT GCA GCA   90
Phe Lys Arg Phe Asp Ala Asn Gly Asp Gly Lys Ile Ser Ala Ala   30

GAG CTT GGG GAG GCC TTG AAA ACA CTT GGC TCC ATC ACA CCG GAT  135
Glu Leu Gly Glu Ala Leu Lys Thr Leu Gly Ser Ile Thr Pro Asp   45

GAG GTG AAA CAT ATG ATG GCT GAG ATT GAC ACC GAT GGC GAC GGC  180
Glu Val Lys His Met Met Ala Glu Ile Asp Thr Asp Gly Asp Gly   60

TTC ATT TCG TTC CAA GAG TTC ACG GAT TTT GCT CGT GCT AAT CGT  225
Phe Ile Ser Phe Gln Glu Phe Thr Asp Phe Ala Arg Ala Asn Arg   75

GGT TTA CTA AAG GAT GTT GCC AAG ATA TTT TAA                  258
Gly Leu Leu Lys Asp Val Ala Lys Ile Phe  *

tgt ctc tgt ctc ttt ctc ttt ttt ggc ata ttt tca tgt cat gat  303
ctc ctt gtt tag agt gat tta ttt tca tgg cta tgg tcc gtt tgg  348
att ttt ctc att ata gaa tta ttt ttg gta gtt cga ctg tca cct  393
ctt gta ttc taa tat caa tgg tgt ttg acc gta tta ttg taa caa  438
gaa aat ggt tca tca aaa aaa aaa aaa aaa a                    469
```

FIG. 1. cDNA and deduced aminoacid sequence of Bet v 4 (clone 6). The two calcium-binding domains are underlined and printed in italics. The 5′ Eco R I site is printed in italics. Nucleotides in the 5′ and 3′ non-coding region are printed in lower case letters while those of the coding region are printed in upper case letters. The cDNA and deduced amino acid sequence of clone 6 have been deposited in the EMBL data base under the accession number Y12560.

RESULTS

Bet v 4 Represents a Two-EF Hand Calcium Binding Birch Pollen Allergen with Homology to Calcium-Binding Allergens from Other Plant Species

A cDNA expression library constructed from mature birch pollen in phage λgt11 was screened with serum IgE from a patient suffering from multivalent plant allergies. According to their IgE-binding pattern clone 6 and 13 appeared to code for novel birch pollen allergens. The cDNA sequence of clone 6 coding for Bet v 4 contained an open reading frame of 255 nucleotides (Figure 1). The protein represents the fourth described birch pollen allergen and was therefore designated Bet v 4. Bet v 4 represents an acidic protein of 9.3 kD deduced molecular weight with a calculated pI of 4.57. Analysis of the sequence of Bet v 4 suggests that this protein will have two canonical EF-hands motifs (32) in the positions indicated in Figure 1. The regions flanking the two calcium-binding loops retain the typical helical periodicity and homology with key residues in calmodulin (Figure 2). The first 10 residues as well as the region between the two EF-hands does not show conservation with other EF-hand sequences and will be expected to be flexible and without fixed conformation. They both contain a large number of hydrophilic or charged residues. The C-terminus is unusually hydrophobic. This region corresponds to the tethering helix in calmodulin and troponin C which links the globular units in a dumbell-like structure. In Bet v 4 this region could either promote interactions with other molecules or dimer formation.

Clone 13 encodes for a fragment of Bet v 4 that lacks the N-terminal 16 amino acids. This would delete the first six residues of the N-terminal helix of the first EF-hand motif. Such a deletion can only destabilize greatly the protein fold with consequent loss of calcium-binding. On the other hand, two mutations found in clone 6 as compared to clone 6 (T in position 156 was silently exchanged to C and C in position 212 was changed to G resulting in a conservative Ala-Gly modification) should not have any effect.

The comparison of the clone 6 DNA sequence with GenBank revealed an almost complete sequence identity with a so far unpublished *Betula verrucosa* mRNA coding for a Bet v 4 pollen allergen submitted under the accession number X87153. The two nucleotides difference in the other submitted sequence were identical to those found in the clone 13 sequence. Clone 6 shared significant average sequence identity of 70% with other two EF-hand pollen proteins from *Brassica napus*, *Brassica rapa* and *Cynodon dactylon* (Figure 2). While the two calcium-binding domains of Bet v 4 were highly homologous with those of other calcium-binding proteins, the N-terminus, C-terminus and the portion between the two calcium-binding sites of Bet v 4 showed significant sequence identity only with the above mentioned two EF-hand pollen proteins.

Recombinant Bet v 4 Binds IgE Antibodies from Approximately 20% of Pollen Allergic Individuals

Recombinant Bet v 4-β-gal fusion protein was obtained by infecting *E. coli* Y1089 with clone 6 phage. Nitrocellulose-blotted *E. coli* extracts containing Bet v 4-β-gal fusion protein and, for control purposes, β-gal alone were probed with sera from 4 representative Bet v 4 allergic patients (Figure 3: lanes 1-4) and from a birch pollen allergic individual without Bet v 4 specific IgE (Figure 3: lane 5). Sera from the Bet v 4 allergic patients displayed IgE reactivity to the Bet v 4-β-gal fusion protein at approximately 125 kD (Figure 3: left panel) while no reactivity with β-gal alone (Figure 3: right panel) was observed. The control serum showed no IgE-reactivity with any protein in both extracts (Figure 3; lanes 5).

In order to determine the frequency of IgE-binding to recombinant Bet v 4 we have tested additional 112 sera from plant allergic patients. Nitrocellulose sectors containing recombinant Bet v 4-β-gal fusion proteins and, for control purposes, β-gal alone were probed with sera from birch pollen-, timothy grass pollen-, olive pollen-, mugwort pollen and maize allergic individuals. Table 1 summarizes the percentage of sera which had

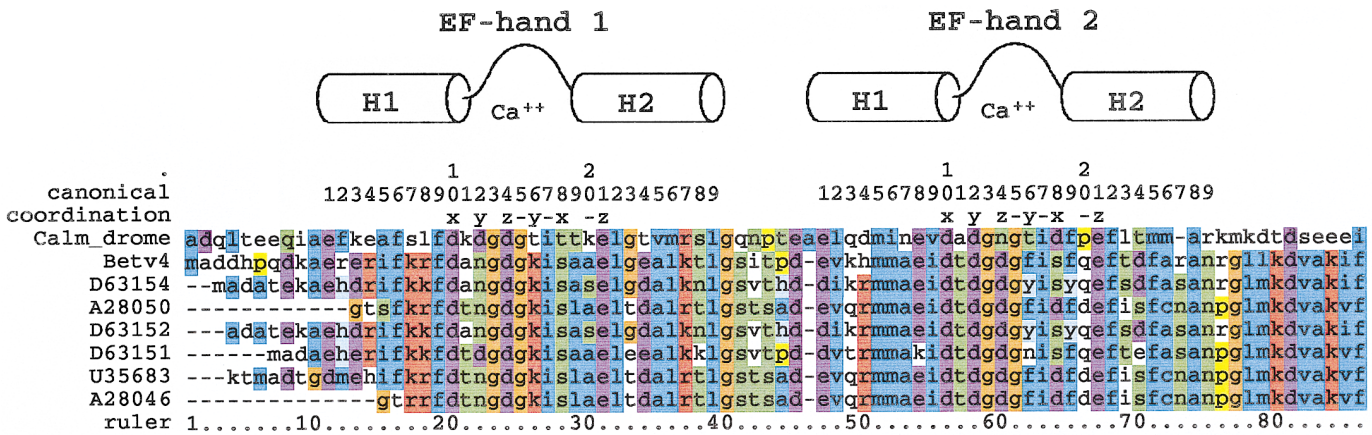

FIG. 2. Multiple sequence alignment of Bet v 4 with other EF-hand proteins. The secondary structure of the two helix-loop-helix EF-hand motifs is indicated on the top. Canonical numbering as proposed by Kawasaki and Kretsinger is reported in the top two lines. The other sequences aligned belong respectively to: Calmodulin (*Drosophila melanogaster*); Bet v 4 (clone 6) (*Betula verrucosa*); D63154: unopened pollen mRNA for calcium-binding protein, *Brassica rapa* bud from EMBL; A28050: B4 clone *Cynodon dactylon* (Bermuda grass) from EMBL; D63152: unopened pollen mRNA for calcium-binding protein, *Brassica napus* (rape) from EMBL; D63151: unopened pollen mRNA for calcium-binding protein, *Brassica napus* (rape) from EMBL; U35683: calcium-binding pollen allergen gene, *Cynodon dactylon* (Bermuda grass) from EMBLNEW; A28046: B1 clone, *Cynodon dactylon* (Bermuda grass) from EMBL.

displayed IgE-reactivity against recombinant Bet v 4. In the group of maize and timothy grass pollen allergic patients we found a lower rate of Bet v 4 sensitization (5-10%) compared to a significantly higher percentage of Bet v 4-reactive sera among the olive and mugwort allergic patients (25-55%). In an average, 20% of the plant-allergic patients displayed IgE-reactivity to recombinant Bet v 4.

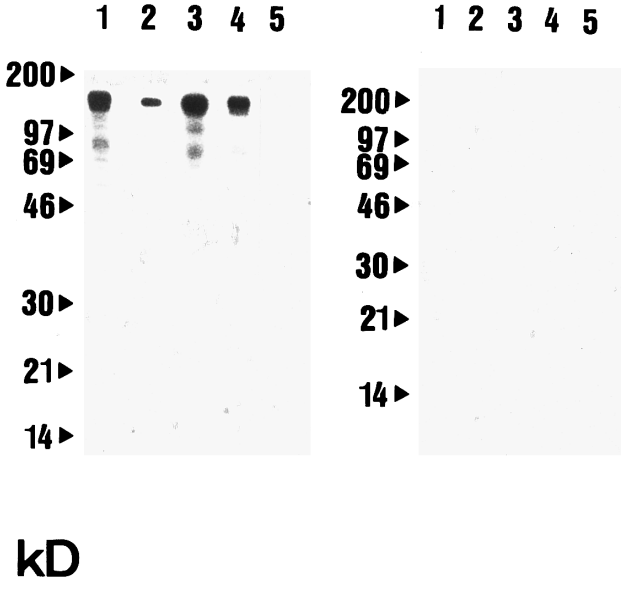

FIG. 3. IgE-binding capacity of recombinant Bet v 4. Nitrocellulose blotted Bet v 4-β-gal fusion protein (left panel) and β-gal alone (right panel) were probed with sera from 4 Bet v 4 allergic individuals (lanes 1-5) and with serum from a birch pollen allergic individual without Bet v 4-specific IgE. Bound IgE antibodies were detected ^{125}I labeled anti-human IgE antibodies. Molecular weight markers are displayed on the left side of each panel.

IgE-Recognition of Bet v 4 Depends on Protein-Bound Calcium; the Bet v 4 N-Terminus Has a Major Contribution to IgE-Binding

Bet v 3, a three EF-hand calcium-binding birch pollen allergen (11), as well as parvalbumins representing major crossreactive fish allergens (33) were reported to contain IgE epitopes that are sensitive to calcium depletion. To determine whether Bet v 4 IgE epitopes require protein-bound calcium, we investigated the IgE-binding capacity of recombinant Bet v 4 in the presence or absence of protein-bound calcium. Nitrocellulose sectors containing complete recombinant Bet v 4-β-gal fusion protein (clone 6), a recombinant Bet v 4 fragment fused to β-gal (clone 13) and for control purposes, recombinant Bet v 2 and Bet v 1-β-gal fusion

TABLE 1

Frequency of IgE-Binding to Recombinant Bet v 4 (clone 6) in Patients Allergic to Different Plant Species

	Percentage of reactive sera				
Clone	Birch (n=12)	Timothy grass (n=20)	Olive (n=40)	Maize (n=20)	Mugwort (n=20)
Clone 6	16%	10%	25%	5%	55%

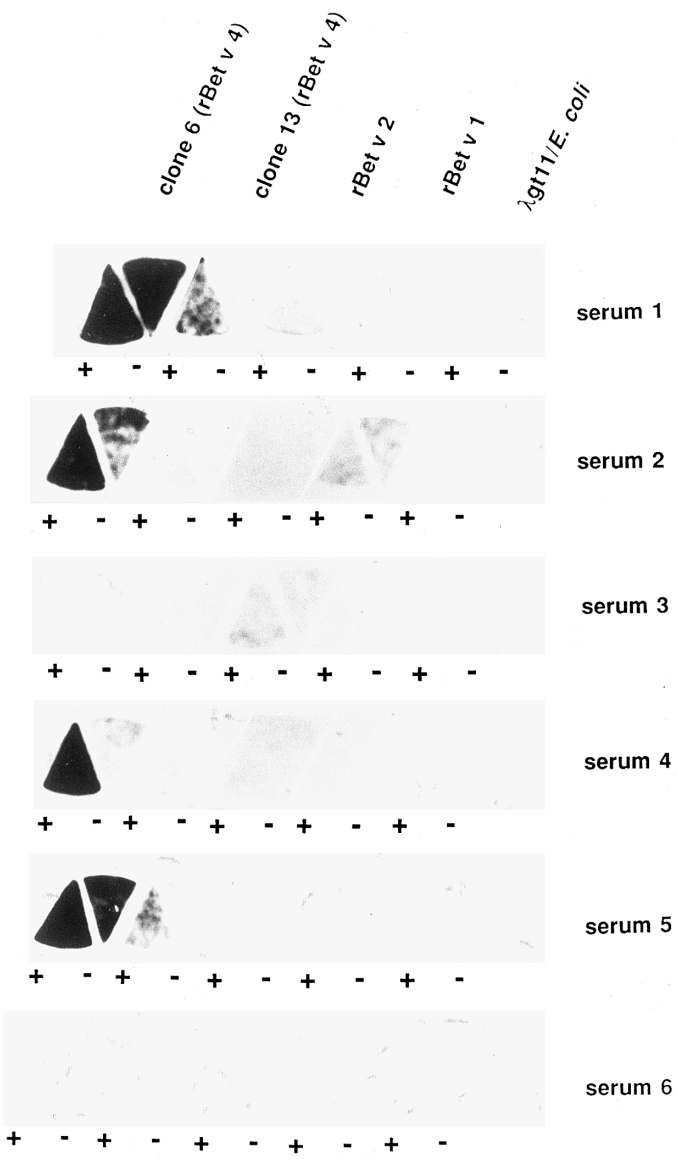

FIG. 4. Effects of calcium-depletion on the IgE-binding capacity of Bet v 4. Nitrocellulose sectors containing recombinant Bet v 4-β-gal fusion protein (clone 6), a recombinant Bet v 4 fragment fused to β-gal (clone 13), recombinant Bet v 2-, recombinant Bet v 1-β-gal fusion proteins and β-gal alone (λgt11/*E. coli*) were probed with serum IgE in the presence of 5 mM $CaCl_2$ (+) or after calcium depletion with 5 mM EGTA (−). Sectors were exposed to sera from Bet v 4-allergic patients (serum 1, 2, 4, and 5), to serum from a profilin allergic individual without Bet v 4-specific IgE (serum 3) and to serum from a non-allergic person (serum 6). Bound IgE was detected with ^{125}I labeled anti-human IgE antibodies and visualized by autoradiography.

proteins as well as β-gal alone were probed with serum IgE from 5 birch pollen allergic patients (Figure 4: sera 1-5) and from a non-allergic individuals (Figure 4: serum 6) in the presence (+) or absence (−) of calcium. Serum IgE from Bet v 4 allergic patients (sera 1, 2, 4 and 5) strongly bound to recombinant Bet v 4 in the presence of calcium. Depletion of protein-bound calcium with EGTA lead to a considerable (24−89%) reduction of IgE binding (Table 2). IgE-recognition of recombinant Bet v 1 by serum 2 and of recombinant Bet v 2 by serum 3 was not influenced by calcium depletion. λgt11/*E. coli* proteins containing β-gal alone were not detected by IgE of any tested serum. The IgE-binding capacity of clone 13 lacking the N-terminal 16 amino acids of Bet v 4 accounted for only between 1−5% of the Bet v 4 IgE-binding capacity. This indicates that either the Bet v 4 N-terminus represents a major IgE-epitope or at least is important for the assembly of a major IgE-binding structure. IgE-recognition of clone 13 also depended on the presence of protein bound calcium. EGTA treatment of clone 13 reduced IgE binding of serum 1 and 5 in the range of 87% and 78%, respectively (Table 2).

Recombinant Bet v 4 Shares IgE-Epitopes with Natural Bet v 4 and a Related Timothy Grass Pollen Allergen

To study whether recombinant Bet v 4 and natural Bet v 4 share IgE-epitopes and whether a timothy grass pollen allergen exists which is immunologically related to Bet v 4, IgE-inhibition experiments were performed. Sera from Bet v 4 allergic patients (A, B) and, for control purposes, serum from a patient allergic to birch and timothy grass pollen containing no Bet v 4-specific IgE, were preadsorbed with recombinant Bet v 4 (Figure 5). Preadsorption of the Bet v 4 positive sera A and B with recombinant Bet v 4 (Figure 5; lanes: +) lead to a complete inhibition of IgE-binding to a protein of approximately 8-9 kD in natural birch pollen extract. Preincubation of the sera with λgt11/*E. coli* proteins (Figure 5; lanes: −) did not effect IgE-binding. IgE binding of serum C to natural Bet v 1 at 17 kD was neither influenced after preincubation with recombinant Bet v 4 nor after preadsorption with λgt11/*E. coli* proteins. Preadsorption of serum B with recombinant Bet v 4 (Figure 5, right panel; lane: +) lead to an almost complete inhibition of IgE-binding to a 8-9 kD timothy grass pollen allergen, while IgE-binding to Phl p 2 at 10 kD and to Phl p 1 isoallergens at approximately 30 kD was not inhibited. IgE reactivity of serum C with Phl p 1 and Phl p 2 was not influenced by preadsorption with recombinant Bet v 4. These data demonstrate that recombinant Bet v 4 shares IgE-epitopes with natural Bet v 4 and a homologous timothy grass pollen allergen of comparable molecular weight.

DISCUSSION

In the present study we report the molecular and immunological characterization of Bet v 4, a 9.3 kD two

TABLE 2

Reduction of IgE-Binding to Bet v 4 after Calcium Depletion; Comparison of the IgE-Binding Capacity of Complete Bet v 4 (Clone 6) and a Bet v 4 Fragment (Clone 13)

	IgE-binding to						Clone 13
	Clone 6			Clone 13			
Patient	Calcium cpm	EGTA cpm	% reduction	Calcium cpm	EGTA cpm	% reduction	% IgE-binding of clone 6
#1	25 080	19 101	24%	1 315	174	87%	5%
#2	16 201	6 046	63%	190	—	100%	1%
#4	5 839	669	89%	212	—	100%	4%
#5	42 131	27 464	35%	565	124	78%	1%

Note. Calcium/EGTA cpm: counts per minute of filter-bound ^{125}I labeled anti-human IgE antibodies after calcium/EGTA treatment. −: no detectable IgE-binding.

EF-hand calcium-binding birch pollen allergen. cDNAs coding for complete Bet v 4 (clone 6) and a Bet v 4 fragment (clone 13) that lacked the first 16 N-terminal amino acids were isolated by screening a birch pollen expression cDNA library with serum IgE from an allergic patient who was sensitized against birch pollen and other plant species. Bet v 4 represents the fourth recombinant birch pollen allergen identified so far. In contrast to Bet v 3 (11), a three EF-hand calcium-binding allergen, Bet v 4 and homologous allergens from weeds (*Brassica*) (17), trees (*Olea europea*) (19) and grasses (*Cynodon dactylon*) (18) contain two copies of EF-hands. EF-hands are helix-loop-helix motifs found in a wide variety of eukaryotic calcium-binding proteins. In the structures determined so far, this motif always occurs in side-to-side pairs of EF-hands which form a globular unit. Each unit can bind up to two calcium ions, one in each helix-loop-helix motif. In canonical EF-hands, such as calmodulin and troponin C, a twelve amino acid residue loop complexes calcium through four carboxylate or carboxamide groups and a single backbone carbonyl oxygen. The liganding oxygens of the residues involved build a pentagonal bipyramid around the calcium ion. Bet v 4 contains two canonical EF-hand motifs with no significant variation from the EF-hand consensus. The two EF-hands are spaced by a hydrophilic linker, six residues long. This linker, as well as the N-terminal sequence is expected to be flexible and unstructured in solution. EF-hands are not new as plant allergens since also Bet v 3, another birch pollen allergen, contained three copies of this motif (11). Although clearly belonging to the EF-hand family, the sequence of Bet v 4, together with its homologous allergens from weeds (*Brassica*), trees (Olea), and grasses (*Cynodon*), represents a distinct sub-family. The close similarity of Bet v 4 with allergens from very distantly related mono- and dicotyledonic plant species (*Brassica napus, Olea europea, Cynodon dactylon*) indicates that the Bet v 4 allergen sub-family may represent a group of highly conserved plant allergens that may elicit allergic crossreactivity in patients suffering from multiple plant allergies. Indeed we observed that approximately 20% of patients suffering from different plant allergies displayed IgE-reactivity against recombinant Bet v 4. Further support for this hypothesis comes from our finding that recombinant Bet v 4 shared

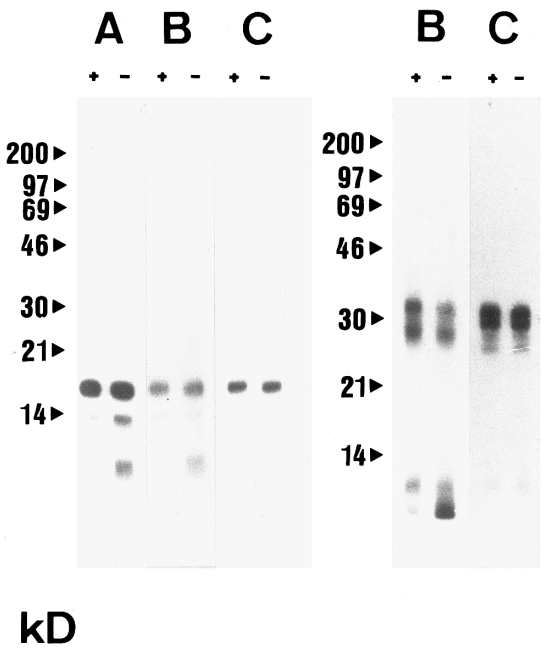

FIG. 5. Recombinant Bet v 4 shares IgE-epitopes with natural Bet v 4 and a Bet v 4-related timothy grass pollen allergen. In the left panel, nitrocellulose-blotted natural birch pollen extract was probed with sera from two Bet v 4 allergic individuals (A, B) and with serum IgE from a Bet v 1 allergic patient without Bet v 4-specific IgE (C). Sera were preadsorbed with recombinant Bet v 4-β-gal (lanes +) or β-gal (lanes −) prior to exposure. Preadsorbed sera B and C (lanes +: Bet v 4; lanes −: β-gal) were also incubated with nitrocellulose blotted timothy grass pollen extract (right panel). Bound IgE-antibodies were detected with ^{125}I labeled anti-human IgE antibodies.

IgE epitopes with a homologous timothy grass pollen allergen of comparable molecular weight. While Bet v 4 and related allergens do not represent major allergens within one plant species, these proteins must be considered as relevant crossreactive plant allergens. As for the profilin allergen family (7, 13), Bet v 4 related allergens are important targets for IgE antibodies of polysensitized patients.

We have previously described that IgE-recognition of allergens that contain calcium-binding domains may depend on the presence of protein-bound calcium. IgE-binding of Bet v 3 (11), a three EF-hand calcium-binding birch pollen allergen, as well as IgE-recognition of parvalbumins which represent major calcium-binding fish allergens (33) was substantially reduced after depletion of protein bound calcium with EGTA. In the present study we demonstrate that IgE recognition of Bet v 4 also requires protein-bound calcium. An up to 90% reduction of IgE-binding to EGTA treated Bet v 4 was observed with certain sera. As for Bet v 3 (11), these results must imply that IgE-antibodies recognize conformational epitopes of Bet v 4 which become accessible only in the calcium-bound conformation. This would be another example of a mode of binding common to the whole EF-hand family. It is well known that EF-hand structures are found in two conformations often referred to as "closed" and "open" (34). Generally, the closed and open conformations correspond to calcium-free and calcium-bound states, respectively. They mainly differ by the relative angles between helices and consequently by a different pattern of helix packing. In the absence of calcium, the four helices would pack in pairs forming a very compact structure (closed state). Upon calcium binding, the two helices of each EF-hand motif are pushed apart inducing the exposure of hydrophobic residues otherwise involved in helix-helix packing in the calcium-free form. These residues become available for interaction with the target protein. A similar mechanism must be valid for the IgE-Bet v 4 recognition. All the residues thought to be essential for the close-to-open transition are present in the Bet v 4 sequence (35). This would then explain the drastic reduction of IgE-binding in the presence of EGTA. It also explains the results found for clone 13, that lacks the first 16 N-terminal amino acids of Bet v 4. While ten of these residues do not show any similarity with other proteins and they should not be part of the EF-hand motif, the following six residues must contribute to the first helix. Deletion of these residues must therefore strongly destabilize the first helix-loop-helix motif and, as a consequence, the second EF-hand which is tightly packed against the first. As a result, calcium binding as well as IgE-binding will be affected. The small residual effect observed for clone 13 in the presence of calcium can be easily explained either by some residual conformation of the fragment or, less likely, by aspecific binding of the several negatively charged residues. Structural analysis of Bet v 4 in the presence or absence of calcium by nuclear magnetic resonance spectroscopy (NMR) and detailed epitope analysis will finally clarify the exact mode of the Bet v 4-IgE-interactin. This analysis may then provide a molecular understanding for the IgE-recognition of Bet v 4 and thus for the IgE-crossreactivity between Bet v 4 and Bet v 4-related allergens. Ultimately this knowledge may help to device molecular approaches for specific therapeutical interventions.

ACKNOWLEDGMENTS

We thank Annalisa Pastore, EMBL, Heidelberg for her valuable suggestions and for critically reading the manuscript. This study was supported in part by Grant S06703 of the Austrian Science Foundation, by a grant of the Austrian Ministry of Science, and by a research grant from Pharmacia, Uppsala, Sweden.

REFERENCES

1. Kaplan, A. P. (1985) Allergy, Churchill Livingstone, New York.
2. Jarolim, E., Rumpold, H., Endler, A. T., Ebner, H., Breitenbach, M., Scheiner, O., and Kraft, D. (1989) *Allergy* **44,** 385–395.
3. Nüchel-Peterssen, B., Janniche, H., Munch, E. P., Wihl, J. A., Böwadt, H., Ipsen, H., and Loewenstein, H. (1988) *Allergy* **43,** 353–362.
4. Valenta, R., and Kraft, D. (1995) *Curr. Opin. Immunol.* **7,** 751–756.
5. Valenta, R., and Kraft, D. (1996) *J. Allergy Clin. Immunol.* **97,** 893–895.
6. Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O., and Breitenbach, M. (1989) *EMBO J.* **8,** 1935–1938.
7. Valenta, R., Duchêne, M., Pettenburger, K., Sillaber, C., Valent, P., Bettelheim, P., Breitenbach, M., Rumpold, H., Kraft, D., and Scheiner, O. (1991) *Science* **253,** 557–560.
8. Larsen, J. N., Stroman, P., and Ipsen, H. (1992) *Mol. Immunol.* **29,** 703–711.
9. Breiteneder, H., Hoffmann-Sommergruber, K., Ebner, C., Reikerstorfer, A., Duchêne, M., Ferreira, F., Valenta, R., Breitenbach, M., Kraft, D., and Scheiner, O. (1992) *J. Allergy Clin. Immunol.* **90,** 927–936.
10. Breiteneder, H., Feirrera, F., Hoffmann-Sommergruber, K., Ebner, C., Breitenbach, M., Rumpold, H., Kraft, D., and Scheiner, O. (1993) *Eur. J. Biochem.* **212,** 355–362.
11. Seiberler, S., Scheiner, O., Kraft, D., Lonsdale, D., and Valenta, R. (1994) *EMBO J.* **13,** 3481–3486.
12. Bufe, A., Spangfort, M. D., Kahlert, H., Schlaak, M., and Becker, W. M. (1996) *Planta* **199,** 413–415.
13. Valenta, R., Duchêne, M., Ebner, C., Valent, P., Sillaber, C., Deviller, P., Ferreira, F., Tejkl, M., Edelmann, H., Kraft, D., and Scheiner, O. (1992) *J. Exp. Med.* **175,** 377–385.
14. Fedorov, A. A., Ball, T., Mahoney, N. M., Valenta, R., and Almo, S. C. (1997) *Structure* **5,** 33–45.
15. Thorn, K. S., Christensen, H. E. M., Shigeta Jr., R., Huddler Jr., D., Shalaby, L., Lindberg, U., Chua, N. M., and Schutt, C. E. (1997) *Structure* **5,** 19–32.
16. Seiberler, S., Scheiner, O., Kraft, D., and Valenta, R. (1992) *Int. Arch. Allergy Immunol.* **99,** 380–381.

17. Toriyama, K., Okada, T., Watanabe, M., Ide, T., Ashida, T., Xu, H., and Singh, M. B. (1996) *Plant Mol. Biol.* **29,** 156–159.
18. Suphioglu, C., Ferreira, F., and Knox, R. B. (1997) *FEBS Lett.* **402,** 167–172.
19. Batanero, E., Villalba, M., Ledesma, A., Puente, X. S., and Rodriguez, R. (1996) *Eur. J. Biochem.* **241,** 772–778.
20. Valenta, R., Duchêne, M., Vrtala, S., Birkner, T., Ebner, C., Hirschwehr, R., Breitenbach, M., Rumpold, H., Scheiner, O., and Kraft, D. (1991) *J. Allergy Clin. Immunol.* **88,** 889–894.
21. Valenta, R., Vrtala, S., Ebner, C., Kraft, D., and Scheiner, O. (1992) *Int. Arch. Allergy Immunol.* **97,** 287–294.
22. Vrtala, S., Grote, M., Duchêne, M., van Ree, R., Kraft, D., and Scheiner, O. (1993) *Int. Arch. Allergy Immunol.* **102,** 160–169.
23. Fling, S. P., and Gregerson, D. S. (1986) *Anal. Biochem.* **155,** 83–88.
24. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
25. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Cold Spring Harbor Laboratory Press.
26. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 680–685.
27. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680.
28. Rost, B., and Sander, C. (1993) *J. Mol. Biol.* **232,** 584–599.
29. Rost, B., and Sander, C. (1994) *Proteins* **19,** 55–72.
30. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
31. Vrtala, S., Sperr, W. R., Reimitzer, I., van Ree, R., Laffer, S., Müller, W. D., Lechner, K., Valent, P., Kraft, D., Scheiner, O., and Valenta, R. (1993) *J. Immunol.* **151,** 4773–4781.
32. Kawasaki, H., and Kretsinger, R. H. (1994) Calcium-binding proteins 1, in: Protein Profile, Academic Press, San Diego.
33. Bugajska-Schretter, A., Elfman, L., Kapiotis, S., Rumpold, H., Valenta, R., and Spitzauer, S. (1997) *J. Allergy Clin. Immunol.* in press.
34. Hertzberg, O., Moult, J., and James, M. N. G. (1986) *J. Biol. Chem.* **261,** 2638–2644.
35. Trave, G., Lacombe, P., Pfuhl, M., Saraste, M., and Pastore, A. *EMBO J.* **14,** 4922–4931.