

Characterization of a birch pollen allergen, *Bet v III*, representing a novel class of Ca^{2+} binding proteins; specific expression in mature pollen and dependence of patients' IgE binding on protein-bound Ca^{2+}

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A cDNA coding for a birch pollen allergen, *Bet v III*, with significant sequence homology to Ca^{2+} binding proteins was isolated from an expression cDNA library using serum IgE from a patient who was allergic to pollen. The deduced amino acid sequence of the pollen allergen contained three typical Ca^{2+} binding sites. Peptides mimicking the Ca^{2+} binding sites of *Bet v III* were synthesized and shown to bind ^{45}Ca in blot overlays. The binding of patients' IgE to the recombinant allergen depended on the native protein conformation and protein-bound Ca^{2+} . Depletion of Ca^{2+} led to a reversible loss of the IgE binding thus representing a conformational IgE epitope adopted by a polypeptide upon Ca^{2+} binding. By RNA hybridization it was demonstrated that *Bet v III* is expressed preferentially in mature pollen. *Bet v III* therefore represents a pollen allergen which because of its unique structural features also belongs to a novel class of Ca^{2+} binding proteins. **Key words:** allergen/*Bet v III*/birch/ Ca^{2+} binding protein/IgE

Introduction

Up to 20% of the population in industrialized countries suffer from Type I allergic symptoms (rhinitis, conjunctivitis and bronchial asthma) (Miyamoto, 1992). As yet only limited information is available regarding the properties of proteins which are capable of inducing an IgE response in man. One example of a well described allergen is profilin, an actin binding protein (Valenta *et al.*, 1991). Due to their biological functions, plant profilins are predominantly expressed in the pollen of different unrelated plant species (Valenta *et al.*, 1992a) and can also be found in other plant tissues albeit in lower amounts (Swododa *et al.*, 1992; Vallier *et al.*, 1992; van Ree *et al.*, 1992; Valenta *et al.*, 1993a). Profilins were subsequently identified as actin binding proteins in higher plants (Rothkegel *et al.*, 1993; Staiger *et al.*, 1993; Valenta *et al.*, 1993b). It has been suggested that profilin is involved in the acrosomal reaction of *Thyone* sperm (Tilney and Inoue, 1982; Tilney *et al.*, 1983) and it is therefore likely that profilins may also play important roles in plant sexual reproduction, in particular in pollen germination which is known to depend critically on the presence of Ca^{2+}

ions (Bednarska, 1989; Obermeyer and Weisenseel, 1991; Miller *et al.*, 1992; Pierson and Cresti, 1992). The intracellular levels and the distribution of Ca^{2+} are tightly controlled by Ca^{2+} binding proteins such as calmodulin and also phospholipids such as inositol trisphosphate (IP_3) which represents a part of a signal transduction cascade involving phosphatidyl inositol bisphosphate (PIP_2) (Heizman and Hunziker, 1991; Trewavas and Gilroy, 1991; Roberts, 1992; Ranjeva *et al.*, 1993) and also the PIP_2 binding protein profilin itself (Goldschmidt-Clermont *et al.*, 1991a,b).

We report now the identification of a birch pollen allergen, designated *Bet v III*, as a Ca^{2+} binding protein. *Bet v III* is expressed specifically in mature pollen and, in contrast to other reported plant Ca^{2+} binding proteins, contains three Ca^{2+} binding motifs. The cDNA coding for *Bet v III* was isolated by expression screening of a birch pollen cDNA library using IgE from allergic patients. Synthetic peptides containing Ca^{2+} binding sites of *Bet v III* bound Ca^{2+} . Moreover, the binding of allergic patients' IgE depended on the native protein conformation and required protein-bound Ca^{2+} . The depletion of Ca^{2+} by EGTA led to a reversible loss of the IgE binding to *Bet v III*. *Bet v III* therefore represents an allergen which belongs to a novel class of Ca^{2+} binding proteins.

Results

Construction of a birch pollen cDNA library; IgE-immunoscreening and analysis of IgE binding phage clones

A cDNA library was constructed from birch pollen mRNA and IgE binding phage clones were isolated with serum IgE from allergic patients (Breiteneder *et al.*, 1989; Valenta *et al.*, 1991). Eleven clones were subsequently tested with IgEs from different patients allergic to birch pollen. As judged by the antibody binding pattern and DNA sequence analyses three clones coded for *Bet v I* (Breiteneder *et al.*, 1989), one clone for birch profilin (Valenta *et al.*, 1991) and two clones for as yet unidentified birch pollen allergens. Four clones (c16, c11, c21 and c13) represented birch pollen allergens with high sequence homology to Ca^{2+} binding proteins. Clones 11, 16 and 21 coded for identical Ca^{2+} binding allergens whereas clone 13 expressed an incomplete fragment of a different Ca^{2+} binding protein. The recombinant allergens expressed by clones 16, 13, 21 and 11 were further tested for their IgE binding capacity with sera from 120 pollen allergic patients. Approximately 10% of the tested patients displayed IgE reactivity with the Ca^{2+} binding allergens.

Characterization of the clone 16 cDNA coding for a Ca^{2+} binding birch pollen allergen (*Bet v III*)

Clone 16 contained the longest cDNA fragment among the Ca^{2+} binding allergens and was therefore selected for

[illegible]

Fig. 1. Comparison of *Bet* v III with other proteins in the EMBL (v.36) and SwissProt databases exhibiting Ca²⁺ binding domains. ATTS1783: *Arabidopsis thaliana* transcribed sequence (Desprez *et al.*, unpublished; accession no. Z26706); STCBP: *Solanum tuberosum* (potato) (Gellatly and Lefebvre, unpublished; accession no. L02830); GMSCAM4: *Glycine max* (soybean) calmodulin (Lee *et al.*, unpublished; accession no. L01433); HVCAMA: *Hordeum vulgare* (barley) calmodulin (Ling and Zielinski, 1989; accession no. M27303). A comparison between the sequences is displayed: 1, ATTS with *Bet* v III and STCBP; 2, *Bet* v III and STCBP; 3, *Bet* v III with ATTS, STCBP, GMSCAM4 and HVCAMA. The Ca²⁺ binding domains are shown in upper case bold; stars indicate identical residues and dots similar ones. The alignment was performed using the program Clustal V (Higgins and Sharp, 1988) with settings of PAM = 250 and indel and floating gap weights of 10. The cDNA and deduced amino acid sequences of *Bet* v III have been submitted to the EMBL and SwissProt databases under the accession number X79267.

detailed characterization. The complete cDNA sequence of clone 16 was determined by sequencing both DNA strands and the amino acid sequence was deduced (Figure 1). The complete cDNA encoded a protein of 203 amino acids with sequence homology to calmodulin from spinach (Lukas *et al.*, 1984), man (Fischer *et al.*, 1988) and *Paramecium tetraurelia* (Schaefer *et al.*, 1987) and to troponin (van Eerd *et al.*, 1978). Sequence homology was also found with a yeast Ca^{2+} binding cell division control protein (Baum *et al.*, 1986) and cod parvalbumin which is known as a relevant fish allergen, *Gad c I* (Elsayed *et al.*, 1974; Elsayed and Bennich, 1975). The sequence homologies found are, however, primarily due to the presence of Ca^{2+} binding sites in the clone 16 sequence (Kretsinger and Nockolds, 1973). Clone 16, which we designated *Bet v III*, contained three Ca^{2+} binding domains (underlined in Figure 1), the carboxy-terminal two having the same structural spacing as those in the calmodulins.

When *Bet v* III was compared with sequences in the EMBL database a more significant homology (53.6% identity and 74.6% similarity) was found to the sequence

of a potato tuber Ca^{2+} binding protein (STCBP, accession no. L02830; Gellatly and Lefebvre, unpublished) which also contained three Ca^{2+} binding domains (Figure 2). The 3'-end of an *Arabidopsis* transcribed sequence (ATTS1783, accession no. Z26706; Desprez *et al.*, unpublished) is also related to the *Bet v* III and potato sequences (Figure 2). These sequences display considerable DNA and protein sequence homology to *Bet v* III outside of the Ca^{2+} binding domains. It is the Ca^{2+} binding domains that account for the sequence homology to the calmodulins, there being little relatedness outside of these domains. CaBP-22, which has a similar molecular weight to *Bet v* III, is a calmodulin with a 47 amino acid extension to its carboxy-terminus (Ling and Zielinski, 1993). It is unrelated to *Bet v* III. The neuronal Ca^{2+} binding proteins hippocalcin, neurocalcin, recoverin, S-modulin and visinin also have approximately the same molecular weight as *Bet v* III and the Ca^{2+} binding domains have the same structural spacing. Again, however, there is little sequence conservation outside the functional Ca^{2+} binding domains (data not shown). All the neuronal Ca^{2+} binding proteins have a non-functional Ca^{2+} binding domain I and, in the case of

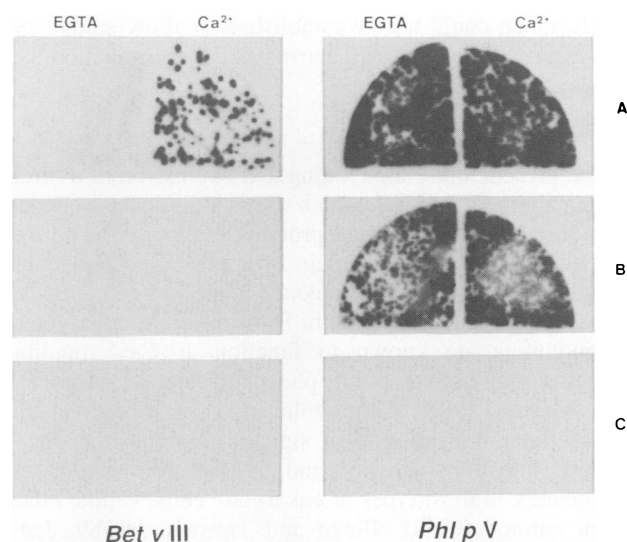


Fig. 2. Dependence of patients' IgE binding to *Bet v III* on protein-bound Ca^{2+} . Nitrocellulose sectors containing native recombinant *Bet v III* or native recombinant *Phl p V* were incubated with serum IgE from allergic patients A and B or with serum IgE from a non-allergic patient C. Patient A displayed IgE reactivity to *Bet v III* and *Phl p V* whereas patient B reacted only with *Phl p V*. The incubation was done with the addition of either 10 mM EGTA (EGTA) or with 10 mM CaCl_2 (Ca^{2+}).

recoverin and S-modulin, domain IV is also modified and does not bind Ca^{2+} . In contrast, only domain II of *Bet v III* appears to be non-functional. In addition, all the reported neuronal Ca^{2+} binding proteins contain a consensus sequence for myristoylation at the amino-terminus. This allows a Ca^{2+} -dependent interaction with membranes (Dizhoor *et al.*, 1993). Such a sequence is absent from *Bet v III* though the first 24 amino acids show little conservation, suggesting they contained targeting information. However, detailed analysis of this sequence did not reveal any obvious structural features or cleavage sites which would suggest a targeting ability and overall the protein was remarkable hydrophilic, suggesting that it is likely to be cytosolic. Hydropathy analysis of *Bet v III* and its homologues showed that they are structurally related (data not shown) and unlike the calmodulins. In conclusion it appeared that *Bet v III* and its homologues might represent a novel class of Ca^{2+} binding proteins which, apart from the Ca^{2+} binding domains, are unrelated to the neuronal Ca^{2+} binding proteins and calmodulins.

Synthetic peptides representing Ca^{2+} binding pockets of *Bet v III* bind Ca^{2+}

The capacity of proteins to bind Ca^{2+} can be demonstrated by blot overlays using ^{45}Ca (Maruyama *et al.*, 1984). According to the deduced amino acid sequence of *Bet v III*, peptides were synthesized which contained the three putative Ca^{2+} binding domains of *Bet v III*. The three peptides, BSA, an unrelated control peptide and spinach calmodulin (controls) were then tested for their ability to bind ^{45}Ca . All three synthetic peptides and spinach calmodulin were able to bind ^{45}Ca whereas no binding was observed to BSA or to the control peptide (data not shown).

IgE binding to *Bet v III* depends on protein-bound Ca^{2+} ; IgE antibodies specific for *Bet v III* can maintain the protein conformation

Recombinant *Bet v III* bound patients' IgE when the protein was tested in non-denaturing plaque assays, whereas the IgE binding capacity was lost when the protein was subjected to denaturing SDS-PAGE and subsequent electroblotting (data not shown). In addition, no protein with a molecular weight corresponding to the calculated molecular weight of *Bet v III* could be detected with patients' IgE in denatured, nitrocellulose-blotted, natural birch pollen extracts (not shown). These data indicated that the binding of allergic patients' IgE might depend on the native protein conformation of *Bet v III*. All IgE binding assays were therefore performed using plaque lifts of non-denatured recombinant proteins.

It was reported earlier that the binding of monoclonal antibodies raised against calmodulin requires protein-bound Ca^{2+} (Hulen *et al.*, 1991). We therefore tested whether the binding of allergic patients IgE is affected by depletion of protein-bound Ca^{2+} using EGTA. Figure 2 shows that depletion of Ca^{2+} from native recombinant *Bet v III* led to a loss of the IgE binding capacity whereas the IgE binding to recombinant *Phl p V* (Vrtala *et al.*, 1993), a major timothy grass pollen allergen, was not affected by the same treatment. To investigate whether the Ca^{2+} binding sites represent IgE epitopes, the peptides were tested for their IgE binding capacity. None of the peptides bound IgE which implies that the IgE binding epitopes are more complex or may be indirectly affected by conformational changes in the Ca^{2+} binding sites.

In a next experiment native recombinant *Bet v III* was tested in four subsequent incubation steps (Figure 3). It is demonstrated that the original loss of the IgE binding by depletion of Ca^{2+} can be restored by subsequent incubation with Ca^{2+} and fresh serum IgE. IgE antibodies which had bound to recombinant *Bet v III* in a buffer containing Ca^{2+} remained bound despite a subsequent incubation with EGTA buffer, indicating that the IgEs are capable of maintaining the protein structure. Using an acidic buffer (100 mM glycine-HCl, pH 2.7) the antibodies were removed and could be rebound in a Ca^{2+} buffer.

Expression of the *Bet v III* RNA in different tissues of birch

In contrast to calmodulin, *Bet v III* appeared to represent a novel class of Ca^{2+} binding proteins with three Ca^{2+} binding domains similar to an unpublished Ca^{2+} binding protein from potato tuber. Pollen germination, particularly the growth of pollen tubes, is known to depend critically on Ca^{2+} (Bednarska, 1989; Obermeyer and Weissenfeld, 1991; Miller *et al.*, 1992; Pierson and Cresti, 1992). This prompted us to investigate the expression of *Bet v III* in different tissues of birch. The RNA blots in Figure 4 showed that the *Bet v III* mRNA is specifically expressed in mature pollen whereas no signal was obtained with RNA from leaves, female inflorescences or male inflorescences harvested at different times. In male inflorescences collected long before liberation of pollen (Figure 4, mI_a) and shortly before liberation of pollen (Figure 4: mI_b, March 24, 1994; mI_c, March 30, 1994; mI_d, April 1, 1994) no *Bet v III* transcript could be detected. Male inflorescences

collected on April 7, 1994 contained no pollen and no hybridization to *Bet v* III transcripts was detected (data not shown). The same blots were then hybridized with an oligonucleotide complementary to the 18S ribosomal RNA of white birch (*Betula verrucosa*) showing that comparable amounts of intact RNA had been transferred to the nitrocelluloses. Although the amount of pollen RNA was lower than in the other RNA preparations, *Bet v* III could be exclusively detected in mature pollen RNA (Figure 4, lane P). Conditions for optimal *in vitro* germination of

birch pollen could not be established to allow studies on the expression of *Bet v* III during pollen germination.

Discussion

In the present study the cloning and expression of a birch pollen allergen, *Bet v* III, which shows significant sequence homology to Ca^{2+} binding proteins is reported. The *Bet v* III cDNA encoded a protein of a calculated molecular weight of 23.7 kDa with sequence homology to calmodulin, troponin and parvalbumin from different eukaryotes. Calmodulins are known to function as Ca^{2+} binding proteins and activators of phosphodiesterase in plants too (Marmé, 1988). Calmodulin also participates in the intracellular transmission of signals involving the phosphatidyl-inositol pathway and is therefore expressed abundantly in most types of eukaryotic cells. Unlike other plant calmodulins (Collinge and Trewavas, 1989; Jena *et al.*, 1989; Ling *et al.*, 1991; Kajiwarra *et al.*, 1992) which possess four typical Ca^{2+} binding domains (I–IV) that probably originated by gene duplication from a smaller ancestor protein, three typical Ca^{2+} binding sites were conserved in the deduced amino acid sequence of *Bet v* III. The results from the sequence comparisons showed that the homologies with calmodulins are primarily due to the EF-hand domains whereas little conservation was found outside the Ca^{2+} binding domains. However, significant sequence homology of *Bet v* III with an unpublished Ca^{2+} binding protein from potato tuber was found which according to hydrophobicity plots is also structurally similar. It is therefore assumed that *Bet v* III belongs to a novel family of Ca^{2+} binding proteins.

To demonstrate that *Bet v* III is able to bind Ca^{2+} , peptides which represent the three Ca^{2+} binding sites of *Bet v* III were synthesized and shown to bind specifically ^{45}Ca in blot overlays.

The structure of Ca^{2+} binding proteins is known to depend on bound Ca^{2+} (Braum and Davis, 1990; Strynadka and James, 1991). Unlike calmodulins, which are rather resistant to denaturation or boiling, *Bet v* III loses its IgE binding capacity when subjected to SDS–PAGE and subsequent electroblotting. We therefore investigated whether the addition or depletion of Ca^{2+} from *Bet v* III might influence the binding of patients' IgE. Using serum IgE and the native recombinant *Bet v* III it is demonstrated

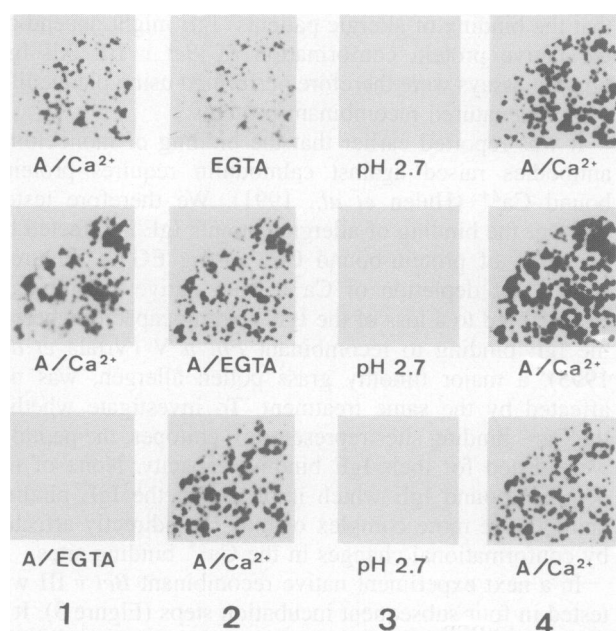


Fig. 3. Reversible loss of patients' IgE-reactivity to *Bet v* III; maintenance of the *Bet v* III conformation by IgE antibodies despite Ca^{2+} depletion. A nitrocellulose sector containing recombinant native *Bet v* III (upper panel) was incubated with serum IgE (A) plus Ca^{2+} (1), then with EGTA (2), subsequently stripped with glycine-HCl, pH 2.7 (3), and finally with serum IgE (A) plus Ca^{2+} (4). The sector in the middle panel was incubated with the same substances in the same order as the sector in the upper panel with the exception that in step 2 serum IgE (A) was added to the EGTA buffer. The lower sector was incubated with serum IgE plus EGTA first (1), then with serum IgE plus Ca^{2+} (2), stripped with glycine-HCl, pH 2.7 (3), and finally incubated with serum IgE plus Ca^{2+} (4). Bound IgE was detected with ^{125}I -labelled rabbit anti-human IgE (Pharmacia, Uppsala, Sweden).

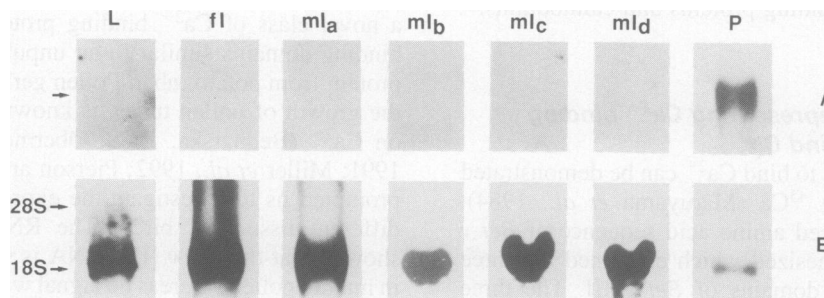


Fig. 4. Specific expression of *Bet v* III in mature birch pollen determined by Northern blotting. 20 μg of total RNA from leaves (L), female inflorescences (fl), from male inflorescences harvested in August (ml_a) and from male inflorescences harvested at various stages before liberation of mature pollen (ml_b , March 24; ml_c , March 30; ml_d , April 1) and from mature pollen (P) were separated in a 1.5% denaturing agarose gel and subsequently blotted onto nitrocellulose. The same membranes were hybridized with the complete *Bet v* III cDNA (A) and with an oligonucleotide complementary to the 18S ribosomal RNA from birch (B). The arrow in A indicates the position of the *Bet v* III transcript at ~800 nucleotides. The position of the ribosomal bands is indicated in B.

that the IgE binding depended on the native protein conformation and required protein-bound Ca^{2+} . Depletion of Ca^{2+} from *Bet v III* using EGTA led to a reversible loss of the allergen's IgE binding capacity which could be restored by subsequent addition of Ca^{2+} and IgEs. Bound IgEs seemed to be capable of maintaining the protein conformation despite incubation with EGTA. This is, to our knowledge, the first example of a conformational IgE epitope adopted by a polypeptide upon Ca^{2+} binding.

Pollen germination is known to depend critically on Ca^{2+} . We therefore speculated that *Bet v III* might fulfil specialized functions in the control of Ca^{2+} metabolism in pollen, which was corroborated by the observation that the mRNA coding for *Bet v III* was expressed preferentially in mature pollen. No mRNA coding for *Bet v III* could be detected in leaves, female inflorescences or male inflorescences containing immature pollen.

The cDNA of *Bet v III* cross-hybridized, however, with mRNA from pollen of other trees belonging to the order Fagales, such as alder, hazel and hornbeam, and we have identified other cDNAs coding for allergens with Ca^{2+} binding motifs from timothy grass pollen (unpublished data). It might therefore be expected that proteins homologous to *Bet v III* are widespread Type I allergens.

The Ca^{2+} metabolism in pollen of higher plants is controlled very tightly by a number of different Ca^{2+} binding proteins among which *Bet v III* appears to represent a novel class of Ca^{2+} binding proteins.

Materials and methods

Materials

Birch (*Betula verrucosa*) pollen was purchased from Allergon, Valinge, Sweden. Leaves, male and female inflorescences were harvested from one birch tree directly into liquid nitrogen and stored at -70°C until use. To follow closely the kinetics of *Bet v III* expression, male inflorescences were collected at short intervals before liberation of mature pollen (March 24, March 30 and April 1, 1994). Male inflorescences harvested on April 7 did not contain any pollen. Sera from allergic patients were characterized as described (Valenta *et al.*, 1992b).

RNA preparation and RNA blots

Total RNA was prepared from 95% pure birch (*Betula verrucosa*) pollen using a phenol–SDS method (Ausubel, 1990). For the preparation of the birch pollen cDNA library poly(A)⁺ RNA was enriched by oligo(dT)–cellulose chromatography (Ausubel *et al.*, 1990). Total RNA from leaves and male and female inflorescences was prepared as described by Dellaporta *et al.* (1983), separated by denaturing agarose gel electrophoresis and blotted onto nitrocellulose. RNA blots were hybridized with the complete cDNA coding for *Bet v III* which was isolated by preparative agarose gel electrophoresis and was labelled with [³²P]dCTP (NEN, Stevenage, UK) according to Feinberg and Vogelstein (1983). All membranes were also hybridized with an oligonucleotide complementary to the 18S ribosomal RNA of birch (Oligo 1: 5'-CTA GAG CTA ATA CGT GCA ACA AAC CCC GAC-3') to ensure that comparable amounts of intact RNA were transferred to the membranes.

Construction and IgE-immunoscreening of a birch pollen cDNA library, DNA sequence analyses and sequence comparisons

A birch pollen expression cDNA library was constructed in phage λ gt11 and screened with serum IgE from a patient allergic to birch pollen (Breiteneder *et al.*, 1989; Valenta *et al.*, 1991). Phage clones expressing birch pollen allergens were characterized with serum IgE from four different birch pollen-allergic patients as described by Valenta *et al.* (1992b). Clones 11, 13, 16 and 21 were further probed with serum IgE from 120 different pollen-allergic patients to determine the frequency of IgE binding.

The cDNAs of clones 11, 13, 16 and 21 were excised from the λ gt11 DNA with *EcoRI*, isolated by preparative agarose gel electrophoresis

(Sambrook *et al.*, 1989) and subcloned into plasmid pUC18 (Boehringer, Mannheim, Germany) in the case of clones 11, 16 and 21 and into plasmid Bluescript SK+ (Stratagene, La Jolla, CA) in the case of clone 13. The cDNA sequence of the first 200 bp was determined using the chain termination method (Sanger *et al.*, 1977) and [³⁵S]dCTP (NEN, Stevenage, UK). Sequences were compared with those in the EMBL (v.36) and SwissProt (v.27) library. Clones 11, 16 and 21 contained identical cDNAs whereas clone 13 represented a cDNA coding for a fragment of a different Ca^{2+} binding protein. The cDNA of clone 16 contained the longest open reading frame and was therefore characterized in detail. Thirty independent phage clones were isolated from the original library with oligonucleotides complementary to the 5'-end of the clone 16 DNA sequence. The amino acid sequence comparisons shown in Figure 1 and the alignments to other Ca^{2+} binding proteins were performed using the program Clustal V (Higgins and Sharp, 1988) with settings of PAM = 250 and indel and floating gap weights of 10. The neuronal Ca^{2+} binding proteins compared with *Bet v III* were in the SwissProt database under the following accession codes: HIPP_RAT, NEC_BOVIN, NECX_BOVIN, RECO_BOVIN, RECO_HUMAN, RECO_MOUSE, SMOD_RAT, VIS1_RAT, VIS2_RAT, VIS3_RAT and VISI_CHICK.

IgE binding assays

Phage expressing the clone 16 cDNA and phage expressing *Phl p V* (Vrtala *et al.*, 1993), a major timothy grass allergen (control), were used to infect *Escherichia coli* Y1090 plated on LB plates containing 100 $\mu\text{g/ml}$ ampicillin (Huynh *et al.*, 1985). Synthesis of the recombinant proteins was induced by overlay with nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) soaked in 10 mM IPTG. The filters containing the recombinant allergens were then cut into sectors and incubated with patients' sera diluted 1:10, and bound IgE-antibodies were detected with a [¹²⁵I]-labelled rabbit anti-human IgE (Pharmacia, Uppsala, Sweden) as described (Valenta *et al.*, 1992b). To investigate the influence of Ca^{2+} on the binding of patients' IgE to *Bet v III*, filters were incubated either in buffer A (50 mM sodium phosphate, pH 7.5, 0.5% Tween 20, 0.5% BSA, 0.05% Na₂S₂O₃) containing 0.1 mM CaCl₂ or in 1 mM EGTA pH 7.5 during the antibody binding assays.

Ca^{2+} binding assays

Three peptides, a: LRLRRIFDLFDKNSDGIITVDELSRA (domain I), b: LRLQGVEDEGDGYISARELQMVLGKLG (domain III) and c: VEKMIVSVDNSNRDGRVDFEFKDMMRVS (domain IV), which contained the three typical Ca^{2+} binding motifs (bold) of clone 16 (*Bet v III*) were synthesized. Purified spinach calmodulin (Sigma, St Louis, MO) was used as a positive control and BSA (Boehringer, Mannheim, Germany) as a negative control. 4 μg of each peptide and the control proteins were dotted onto nitrocellulose. Nitrocelluloses were then incubated with ⁴⁵Ca (NEN, Stevenage, UK), washed and exposed as described (Maruyama *et al.*, 1984).

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