

Molecular and Structural Analysis of a Continuous Birch Profilin Epitope Defined by a Monoclonal Antibody*

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The interaction of a mouse monoclonal antibody (4A6) and birch profilin, a structurally well conserved actin- and phosphoinositide-binding protein and cross-reactive allergen, was characterized. In contrast to serum IgE from allergic patients, which shows cross-reactivity with most plants, monoclonal antibody 4A6 selectively reacted with tree pollen profilins. Using synthetic overlapping peptides, a continuous hexapeptide epitope was identified. The exchange of a single amino acid (Gln-47 → Glu) within the epitope was found to abolish the binding of monoclonal antibody 4A6 to other plant profilins. The NMR analyses of the birch and the nonreactive timothy grass profilin peptides showed that the loss of binding was not due to major structural differences. Both peptides adopted extended conformations similar to that observed for the epitope in the x-ray crystal structure of the native birch profilin. Binding studies with peptides and birch profilin mutants generated by *in vitro* mutagenesis demonstrated that the change of Gln-47 to acidic amino acids (e.g. Glu or Asp) led to electrostatic repulsion of monoclonal antibody 4A6. In conclusion the molecular and structural analyses of the interaction of a monoclonal antibody with a continuous peptide epitope, recognized in a conformation similar to that displayed on the native protein, are presented.

To study the mode of the interaction of protein antigens with their antibodies, defined experimental systems are required. In those cases in which crystal structures of antibodies with their corresponding antigen have been determined, it was found that the epitopes (antigenic determinants) belonged to the discontinuous type of epitopes, i.e. several surface loops are involved in the interaction with the corresponding paratope (Amit *et al.*, 1986; Sheriff *et al.*, 1987; Padlan *et al.*, 1989; Tulip *et al.*, 1990; reviewed in Berzofsky, 1985; Braden and Poljak, 1995). In contrast, it has been proposed that epitopes on native proteins consist mainly of short sequence segments of about 6 amino

acids that can be mimicked by utilizing synthetic peptides (Green *et al.*, 1982). Indeed, it was demonstrated that small peptides can elicit antibodies with sequence and structural requirements for binding antigens comparable to antibodies raised against the native protein (Geysen *et al.*, 1985) and that overlapping oligopeptides can be used for epitope analysis (Geysen *et al.*, 1987). Despite these data, the existence of epitopes consisting of small continuous sequence motifs in native proteins has been questioned with the argument that antibodies elicited against peptides might selectively react with denatured, unfolded proteins (Jemmerson and Blankenfeld, 1989). In this context, we studied the interaction of a structurally well defined protein antigen with a monoclonal antibody. We used birch pollen profilin as a model (Valenta *et al.*, 1991). Profilins are small (14–17 kDa) proteins found in all eukaryotic phyla that bind to actin and to polyphosphoinositol lipids, particularly to phosphatidylinositol 4,5-bisphosphate, and thus may represent a link between the cytoskeleton and signal transduction (Machesky and Pollard, 1993; Sohn and Goldschmidt-Clermont, 1994; Drobak *et al.*, 1994). In addition all profilins bind to poly-L-proline (Tanaka and Shibata, 1985; Kaiser *et al.*, 1989; Schutt *et al.*, 1993; Björkegren *et al.*, 1993; Archer *et al.*, 1994; Metzler *et al.*, 1994). Recently, the first biologically relevant proline-rich ligand for profilin was identified (Reinhard *et al.*, 1995).

Profilins have also been described as potent allergens (Valenta *et al.*, 1991; 1992; Vallier *et al.*, 1992). IgE antibodies from profilin-allergic patients were shown to cross-react with profilins from different sources, which has led to the designation of profilins as “pan-allergens” (Valenta *et al.*, 1992). In the present study we have analyzed the interaction of birch profilin with a specific mouse monoclonal antibody at the molecular and structural level.

mAb¹ 4A6 bound to a continuous hexapeptide epitope that, according to the comparison of the peptide NMR analysis and the crystal structure of birch profilin, formed a similar conformation as in the native protein. Gln-47 was determined as the crucial amino acid for the contact with mAb 4A6 using structural data, peptide variants, and protein mutants.

EXPERIMENTAL PROCEDURES

Preparation of Pollen Extracts from Different Plant Species—Pollen from white birch (*Betula verrucosa*), alder (*Alnus glutinosa*), tobacco

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y08364.

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¹ The abbreviations used are: mAb, monoclonal antibody; HPLC, high pressure liquid chromatography; ELISA, enzyme-linked immunosorbent assay; CDR, complementary determining region; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; TOCSY, total correlation spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy.

(*Nicotiana tabacum*), mugwort (*Artemisia vulgaris*), timothy grass (*Phleum pratense*), maize (*Zea mays*), and wheat (*Hordeum vulgare*) was purchased from Allergon, AB (Välinge, Sweden). The pollen preparations were pure, as judged by light microscopy. 3 g of pollen were homogenized with an ultraturrax (Ika, Germany) in 50 ml of homogenization buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 5 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine) and further extracted under continuous agitation at 20 °C for 2 h. Supernatants were obtained by centrifugation at 19,000 rpm at 4 °C for 30 min, dialyzed against water, and lyophilized. Lyophilized extracts were stored at -20 °C until use.

Expression and Purification of Recombinant Birch Profilin—Recombinant birch profilin was expressed in *Escherichia coli* BL21 (DE3) (Studier *et al.*, 1990) using a T7 RNA polymerase system and plasmid pMW175prof (Susani *et al.*, 1995), which contained the cDNA coding for birch profilin (Valenta *et al.*, 1991). Expression and purification of the recombinant protein were performed as described (Giehl *et al.*, 1994). Recombinant birch profilin was dialyzed against CMF-PBS (136 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.2) and stored at 4 °C.

Peptides—The following peptides were purchased from Chiron Mimotope Peptide Systems and supplied as >90% pure peptides: PQFKPQ, PQFKPE, SSSFPQFKPQEITG, SASFPQFKPEEITG, and SSTFPKFKPEEITG. SFPQFKPQEITG and SFPQFKPEEITG for NMR analysis were purified by HPLC to >95% purity. Peptides SFPQFKPQEITG, SFPQFKPEEITG, SFPQFKPNEITG, SFPQFKPDEITG, and SFPQYKPQEITG containing changes in position 44 and 47 were synthesized using the Multipin Peptide Synthesis System (Geyssen *et al.*, 1984). All peptides contained an amidated carboxyl terminus and an acetylated amino terminus. The peptide BP36/51 (AQSSFPQFKPQEITG) was synthesized on an automated synthesizer (Milligen, model 9050). Synthesis and deprotection followed standard protocols of the manufacturer. The peptide was purified by preparative HPLC on a Vydac 218 Tp 1022 column, and its calculated molecular mass was verified by matrix-assisted laser desorption ionization mass spectrometry.

Antibodies—The mouse monoclonal antibody 4A6 was raised against purified recombinant birch profilin by immunizing female BALB/c mice with Titer-Max (Hunter). Mouse spleen cells were fused with the Ag8 myeloma line as described (Geffert *et al.*, 1977). The hybridoma clone producing mAb 4A6 was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies, Inc.).

Rabbit antibodies (RP) were raised against purified celery root profilin (RP1), recombinant birch profilin (RP2), and a synthetic 25-mer carboxyl-terminal peptide of the birch profilin carboxyl terminus that had been coupled to keyhole limpet hemocyanin (RP3) (Vallier *et al.*, 1992; Valenta *et al.*, 1993).

Characterization of mAb 4A6 by Sequence Analysis—RNA was isolated from 4A6 hybridoma cells using the guanidine isothiocyanate method and CsCl gradient centrifugation (Davis *et al.*, 1986). cDNA coding for the heavy and light chain of the 4A6 antibody was reverse-transcribed and amplified by PCR using a RNA-PCR kit (Perkin Elmer). The following oligonucleotides (Biomedica, Vienna, Austria) were used as primers: heavy chain constant and variable regions, 5' AGG CTT ACT AGT ACA ATC CCT GGG CAC AAT 3' and 5' AG GTC CAG CTG CTC GAG TCT GG 3'; for the light chain constant and variable regions, 5' T CCT TCT AGA TTA CTA ACA CTC TCC CCT GTT 3' and 5' GT GCC AGA TGT GAG CTC GTG ATG ACC CAG TCT CCA 3'.

Amplified cDNAs coding for the heavy chain fragment and light chain of mAb 4A6 were then purified by preparative agarose gel electrophoresis and cut with *SpeI/XhoI* and *SacI/XbaI*, respectively (Sambrook *et al.*, 1989). The DNA fragments were again purified by agarose gel electrophoresis and subcloned into plasmid pComb 3 (Barbas *et al.*, 1991). The sequence of both heavy chain fragment and light chain cDNA was determined by primer walking (Sanger *et al.*, 1977) using ³⁵S-labeled dCTP (DuPont NEN) and a T7 sequencing kit (Pharmacia Biotech Inc.).

Immunoblotting and Dot Blots—Protein extracts were subjected to denaturing polyacrylamide electrophoresis according to Laemmli (1970) and then blotted onto nitrocellulose as described (Towbin *et al.*, 1979). For dot blots, approximately 50 ng of each peptide were applied to nitrocellulose membranes. Nitrocelluloses were incubated with rabbit or mouse antibodies diluted in buffer A (50 mM sodium phosphate, pH 7.5, 0.5% Tween 20, 0.5% bovine serum albumin, and 0.05% Na₂S₂O₃). Bound mouse antibodies were detected with a 1:500-diluted ¹²⁵I-labeled sheep anti-mouse antiserum (Amersham Corp.). Bound rabbit antibodies were detected with a 1:2000-diluted ¹²⁵I-labeled donkey anti-rabbit

antiserum (Amersham Corp.). Dried filters were exposed to Kodak X-Omat S films for approximately 48 h using intensifying screens (Eastman Kodak Co.).

Competitive ELISA—Competitive ELISAs were done as described by Wehland *et al.* (1984). ELISA plates (Nunc, Roskilde, Denmark) were coated with 100 ng of purified recombinant birch profilin dissolved in 50 µl of CMF-PBS overnight at 4 °C. The plates were then washed three times with CMF-PBS and saturated with 200 µl of 4% bovine serum albumin in CMF-PBS/well for 2 h at 37 °C. 50 ng of protein G purified 4A6 antibody/well and different concentrations of recombinant birch profilin or synthetic peptide BP36/51 were mixed and preincubated at 30 °C for 30 min, then 50 µl of the mixture were added to each well and incubated for an additional 2 h at 37 °C. The plates were then washed five times with CMF-PBS, and bound monoclonal antibodies were detected with horseradish peroxidase-labeled rabbit anti-mouse Ig (Sigma) diluted 1:3000 in CMF-PBS (Engvall and Perlmann, 1972). The absorbance was measured at 490 nm using an ELISA reader (Microplate Autoreader EL 310, Biotek Instruments). Triplicate assays were performed for each dilution, and the average values of 10 measurements are displayed.

Site-directed Mutagenesis of Birch Profilin—Birch profilin mutants were generated using plasmid pMW175prof (Susani *et al.*, 1995) according to the method of Deng and Nickoloff (1992), using the selection primer 5' CAT CAT TTG GGG CTG GTA CCC GAT CCG GCT GC 3' containing a *KpnI* site (italics) and the following mutagenic primers: Y44, 5' CTT CCT TCC CAC AGT ATA AGC CTC AGG AAA TC 3'; E47, 5' CCA CAG TTT AAG CCT GAG GAA ATC ACT GGT ATC 3'; and N47, 5' CCA CAG TTT AAG CCT AAC GAA ATC ACT GGT ATC 3' (triplets in italic code for the changed amino acid). The primers were purchased 5'-phosphorylated and fast protein liquid chromatography-purified (Pharmacia). *In vitro* mutagenesis was done using a Chameleon™ double-stranded site-directed mutagenesis kit (Stratagene, La Jolla, CA) and *E. coli* XLmutS strain Δ(*mcrA*)183 Δ(*mcrCB*-*hsdSMR*-*mrr*)173 *endA1* *supE44* *thi-1* *gyrA96* *relA1* *lac mutS*::Tn10(Tet^r) [*F'*proAB *lacI*^qΔ*M15* Tn5 (Kan^r)]. The sequence of the mutagenized clone 4, clone 25, and clone 35 was confirmed by sequencing both DNA strands using the following primers: forward primer, 5' TAA TAC GAC TCA CTA TAG GG 3'; reverse primer, 5' GCT AGT TAT TGC TCA GCG G 3'. Plasmids pMW175prof, pMW175profY44, pMW175profE47, and pMW175profN47 were then transformed into *E. coli* K12 CAG748 *thi leu lacYtonAsupB44* Δ(*lac*)X90 *dnaJ259 thr*::Tn10 (Straus *et al.*, 1988), which was cotransformed with plasmid pT7POL22 (LMBP Culture Collection, Gent, Belgium) for high level protein expression (Mertens *et al.*, 1995).

Calculation of Probable Conformations of the 4A6 Birch Profilin Epitope and the Homologous Peptides from Other Plants—For the calculation of probable backbone conformations of the birch profilin epitope and the homologous peptides from maize, timothy grass, tobacco, and wheat, the Boltzmann device was used (Sippl, 1990). The overall structures were then built from the ensembles as described (Sippl *et al.*, 1992).

NMR Analysis of the Birch Profilin and Timothy Grass Profilin Peptides; Superimposition with the Birch Profilin Crystal Structure—Samples of 5 mg of each peptide were dissolved in 0.5 ml of 10 mM potassium phosphate, pH 7.0, in 10% D₂O, 90% H₂O. Chemical shifts were referenced to trimethylsilylpropionate. All data were collected at 10 °C on a Bruker DMX-600. Magic-angle gradient double-quantum-filtered COSY spectra (van Zijl *et al.*, 1995) were collected as 512 *t*₁ increments of 16 scans each. TOCSY spectra (Bax and Davis, 1985) with a 70-ms mixing time were collected as 512 *t*₁ increments of 8 scans each, and ROESY spectra (Griesinger and Ernst, 1987) with a 300-ms mixing time were collected as 512 *t*₁ increments of 32 scans each. Quadrature detection in *t*₁ was achieved by the time proportional phase incrementation method (Marion and Wüthrich, 1983). Water suppression in the TOCSY and ROESY experiments was performed by the double-pulsed field gradient echo technique (Hwang and Shaka, 1995). All spectra were processed with NMRPipe (Delaglio *et al.*, 1995). Resonance assignments were made by standard procedures (Wüthrich, 1986). Conformations of the birch and timothy grass peptide were calculated by distance geometry with the program DIANA (Güntert *et al.*, 1991), using a total of 47 inter-residue rotating frame Overhauser effect distance restraints for the timothy peptide and 58 inter-residue rotating frame Overhauser effect distance restraints for the birch peptide. Upper distance bounds for the restraints were set to 5 Å, and 10 conformations that satisfied the distance restraints were calculated for each peptide.

The conformation of the 4A6 hexapeptide epitope in the native protein was derived from the x-ray crystal structure of birch profilin

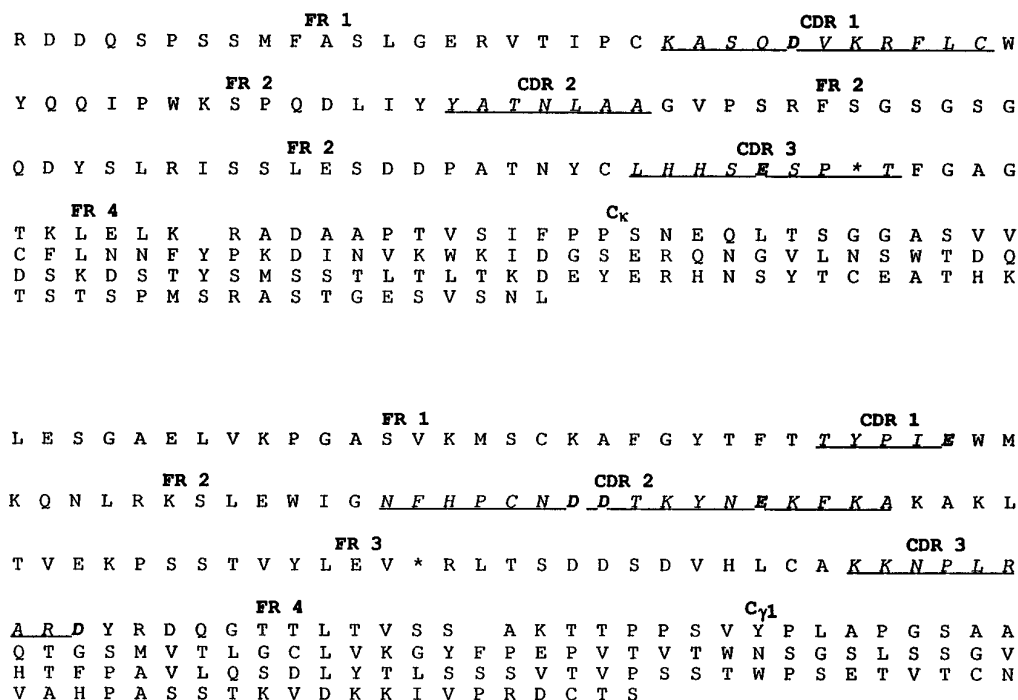


FIG. 1. **Deduced amino acid sequence of heavy chain fragment and light chain of the 4A6 Fab.** The deduced amino acid sequence of the 4A6 light chain (top) and heavy chain fragment (bottom) is displayed. The framework regions (FR) and constant regions are indicated. The CDRs are underlined and acidic amino acid residues are printed in **bold type**. The heavy chain fragment contained one point mutation introduced by the PCR amplification, which led to the insertion of a stop codon in FR3 (*). A PCR-based mutation based on the insertion of one base in CDR3 of the light chain is also indicated by an asterisk. The cDNA and deduced amino acid sequences of the 4A6 Fab were deposited in the EMBL nucleotide sequence data base under the accession number Y08364.

determined at 2.4-Å resolution.² Surface accessibility calculations were performed with the analytic method of Richmond using a probe size of 1.4 Å.

RESULTS AND DISCUSSION

The interaction of birch pollen profilin and a specific mouse monoclonal antibody, designated 4A6, was investigated. Birch profilin (Valenta *et al.*, 1991; 1992; 1993) was chosen as a model for antigen-antibody interactions for two reasons. First, although they display only modest sequence homology, profilins are structurally well conserved eukaryotic proteins, which may be due to their conserved function as actin-binding proteins (Almo *et al.*, 1994; Fedorov *et al.*, 1994). Indeed it could be shown that despite a low degree of sequence similarity, profilin and actin from different species could interact *in vitro* as well as *in vivo* (Valenta *et al.*, 1993; Giehl *et al.*, 1994; Staiger *et al.*, 1994; Rothkegel *et al.*, 1996). Additionally, profilins are potent allergens that induce cross-reactive IgE antibodies in about 20% of allergic patients (Valenta *et al.*, 1991).

mAb 4A6 consists of an IgG₁ heavy chain and a κ light chain. The deduced amino acid sequence of the 4A6 amino-terminal heavy chain fragment and its corresponding light chain are shown in Fig. 1. In the CDRs of the light chain two acidic amino acids were found, whereas in the CDRs of the heavy chain five acidic amino acids were observed. Despite a high degree of sequence identity of approximately 80% among profilins from higher plants, mAb 4A6 was able to discriminate between tree pollen profilins and other plant profilins (Fig. 2). The 4A6 epitope was mapped using synthetic dodecapeptides that spanned the deduced amino acid sequence of birch profilin by 10 amino acids of overlap. mAb 4A6 bound strongly to peptides (amino acids 38–49 and amino acids 40–51) of birch profilin, whereas peptides (amino acids 36–47 and amino acids 42–53)

reacted more weakly. All peptides reacting with 4A6 shared the 6-amino acid sequence motif PQFKPQ. This sequence motif was compared with the relevant region in other plant profilins (Staiger *et al.*, 1993; Valenta *et al.*, 1994; Rihs *et al.*, 1994; Mittermann *et al.*, 1995). The only consistent sequence difference between birch profilin and the other plant profilins was seen in the last position of the hexapeptide. Here, only birch profilin contained Gln-47 instead of Glu.

The difference in binding of mAb 4A6 to different plant profilins was further investigated at the epitope level using peptides corresponding to the birch, timothy grass, and tobacco epitope. Peptides comprising 14 amino acids of the plant profilins were probed for binding to mAb 4A6 by dot blotting (Fig. 3). 4A6 did not bind to the timothy grass and tobacco peptides but reacted with the birch epitope peptides synthesized as 14-mer, 12-mer, and hexapeptide. The binding intensity decreased with the length of the peptides. Thus, as the minimal epitope for mAb 4A6, the birch hexapeptide PQFKPQ was identified, which differed from the other plant profilin peptides by a single amino acid (Gln-47 → Glu).

To compare the affinity of recombinant birch profilin with a synthetic peptide epitope spanning amino acids 36–51, competitive ELISA studies were performed (Fig. 4). Purified recombinant birch profilin was coated to ELISA plates and probed with mAb 4A6 that was preincubated either with purified recombinant birch profilin or the synthetic birch profilin peptide BP36/51. The concentration for a 50% competition with recombinant birch profilin was determined to be 1.2×10^{-7} M for recombinant birch profilin and 5×10^{-8} M for the peptide BP36/51, when 50 ng of purified mAb 4A6 were used per well. Thus, the peptide BP36/51 displayed a slightly higher affinity for mAb 4A6 than the complete recombinant birch profilin.

Hence, the 4A6 epitope represents a continuous epitope, a term coined for peptide epitopes consisting of short sequence motifs (Berzofsky, 1985). Although continuous epitopes have

² A. A. Fedorov, T. Ball, N. Mahoney, R. Valenta, and S. C. Almo, submitted for publication.

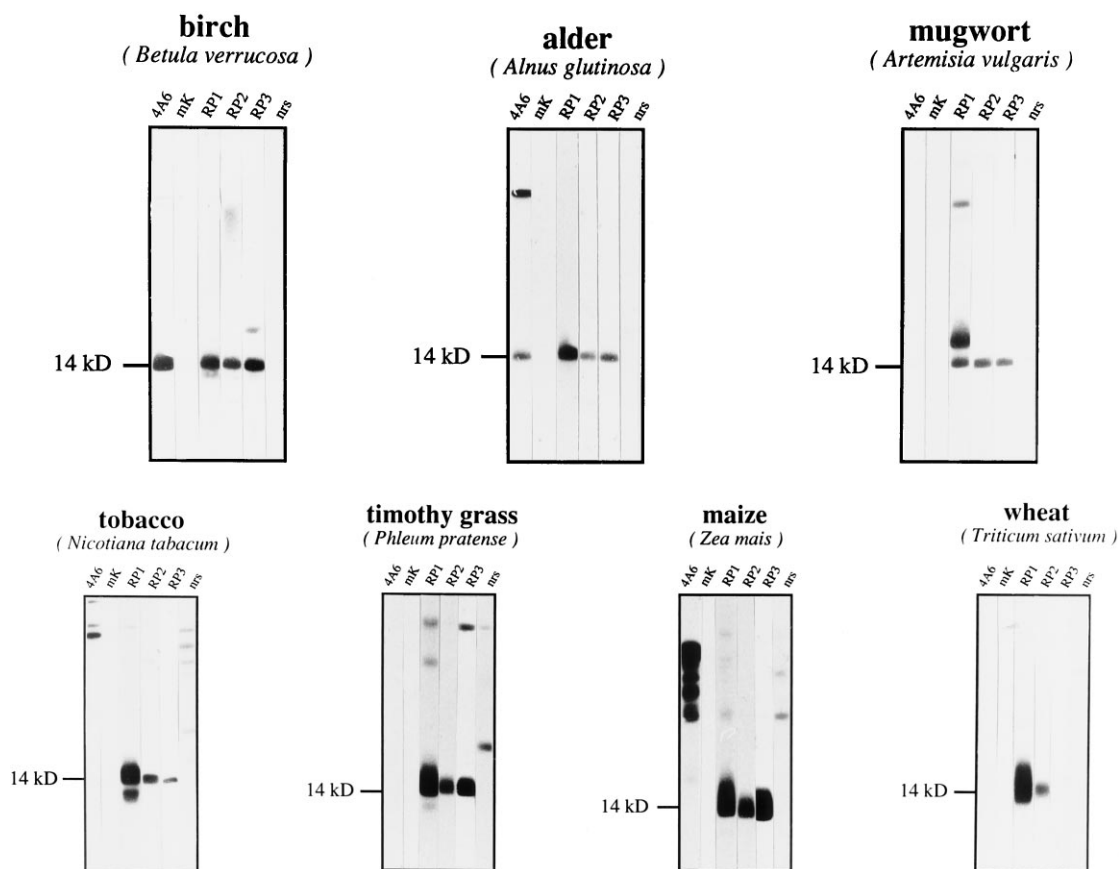


FIG. 2. Reactivity of mAb 4A6 and anti-plant profilin antisera with nitrocellulose-blotted profilins from different plant species. Protein extracts were prepared from pollens of different plant species, comprising dicotyledonic plants (birch, *Betula verrucosa*; alder, *Alnus glutinosa*; mugwort, *Artemisia vulgaris*; tobacco, *Nicotiana tabacum*) and monocotyledonic plants (timothy grass, *Phleum pratense*; maize, *Zea mais*; wheat, *Triticum sativum*), separated by SDS-polyacrylamide gel electrophoresis, and blotted to nitrocellulose. Nitrocellulose strips were then incubated with the mouse monoclonal anti-birch profilin antibody (4A6), a mouse monoclonal antibody without specificity for profilins (mK), a rabbit antiserum raised against celery root profilin (RP1), a rabbit antiserum raised against recombinant birch profilin (RP2), a rabbit antiserum raised against the birch profilin carboxyl terminus (RP3), and a normal rabbit serum (nrs). Antibodies bound to profilin at 14 kDa were detected with a ^{125} I-labeled sheep anti-mouse and donkey anti-rabbit antiserum, respectively.



FIG. 3. Reactivity of mAb 4A6 with peptides from different plant profilins tested by dot blotting. In lane 1 peptides were tested with a monoclonal antibody without specificity for birch profilin, and in lane 2 mAb 4A6 was used. Approximately 100 ng of each peptide were dotted to the nitrocellulose in the order shown.

been reported for a number of antigens, and antibodies were described that bound with comparable affinity to a peptide epitope and the complete native protein (Navon *et al.*, 1995; Fernandez *et al.*, 1994), the physiological role of continuous epitopes has been questioned (Laver *et al.*, 1990).

Crystallographic analyses of antigen-antibody complexes of intact proteins demonstrated that binding predominantly involves conformational epitopes, which are assembled from multiple peptide segments separated in the primary sequence (reviewed in Braden and Poljak, 1995). Such conformational

epitopes have been described for other birch pollen allergens. A calcium-binding birch pollen allergen, Bet v 3, contained an epitope that was sensitive to depletion of calcium and denaturation (Seiberler *et al.*, 1994). IgE epitopes of the major birch pollen allergen Bet v 1 (Breiteneder *et al.*, 1989) could not be determined with overlapping peptides, and protein fragments did not demonstrate IgE antibody binding.³

In order to obtain information whether the different binding of mAb 4A6 to the plant peptides might be due to conformational differences, the protein backbone conformations for the birch, maize, timothy grass, tobacco, and wheat peptides were calculated from the data base with the Boltzmann device (Sippl, 1990), revealing a rather similar structure for the different peptides (data not shown). The prediction was confirmed by NMR analysis of the birch (SFPQFKPQEITG) and timothy (SFPQFKPEITG) peptide. Both peptides showed extended conformation in solution (Fig. 5). The alignment of the ensemble of NMR structures calculated for the birch P3-Q8 peptide segment gave a consistent set of structures, whereas the timothy P3-E8 peptide segment displayed more variability, due to fewer and weaker nuclear Overhauser effects.

The x-ray structure of birch profilin, determined at 2.4-Å resolution,² also showed that the 4A6 epitope adopted an extended conformation in the native birch profilin molecule.

³ S. Vrtala, K. Hirtenlehner, L. Vangelista, A. Pastore, H.-G. Eichler, W. R. Sperr, P. Valent, C. Ebner, D. Kraft, and R. Valenta, submitted for publication.

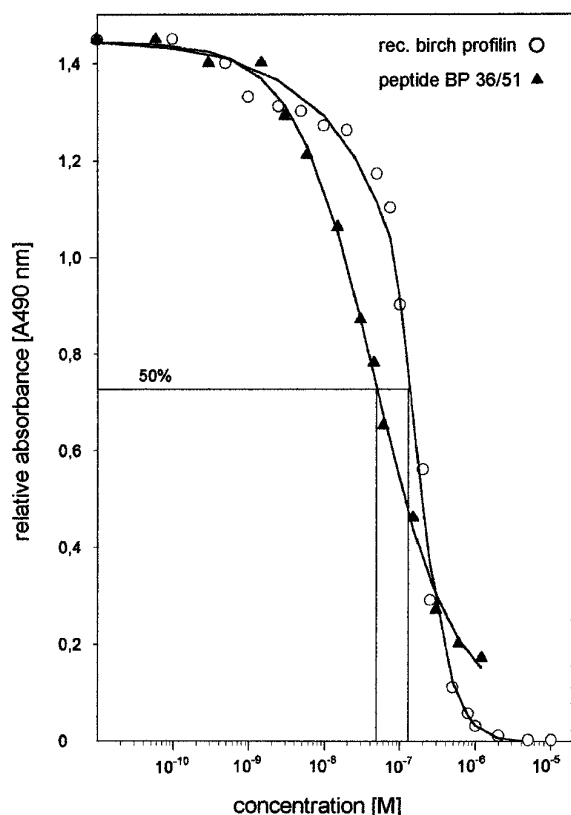
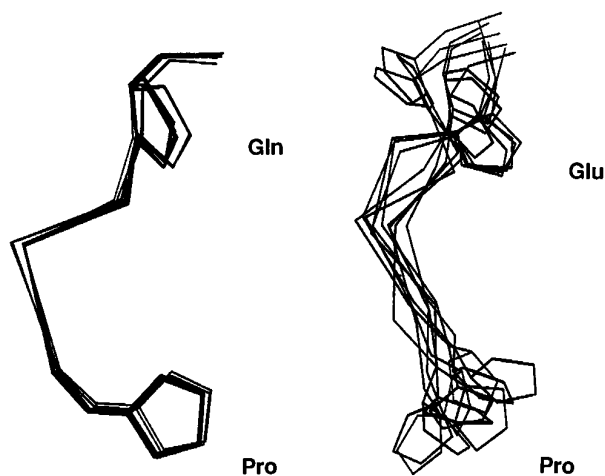


FIG. 4. **ELISA competition assay.** ELISA plates were coated with 100 ng of recombinant birch profilin/well. Purified mAb 4A6 was allowed to bind to recombinant birch profilin using increasing concentrations (x axis) of recombinant birch profilin (○) or peptide BP 36/51 (▲) as competitor for preincubation. The extinction is displayed as relative absorbance at 490 nm on the y axis.



birch: S F P Q F K P Q E I T G timothy: S F P Q F K P E E I T G

FIG. 5. **Superimposition of the 10 best NMR structures of the QE (birch, left) and EE (timothy, right) peptides.** The C α traces, along with proline side chains, are shown for the residues corresponding to positions 42–47 of the birch profilin sequence. The structures were aligned to minimize the root mean squared deviations between the positions of equivalent backbone atoms.

When the peptide was considered in the context of the folded protein (Table I) a significant burial of surface area is seen for only two amino acids. Pro-42 and Phe-44 have a large buried surface area due to extensive packing in the hydrophobic core of birch pollen profilin. In contrast, Gln-43, Lys-45, Pro-46, and

TABLE I
Solvent accessibility of the 4A6 epitope in the protein background and in isolation

Amino acid	Accessibility in intact protein	Accessibility in isolated peptide	Buried surface ^a	Buried ^b
	Å ²	Å ²	Å ²	%
Pro-42	40	181	141	78
Gln-43	146	176	30	17
Phe-44	23	168	145	86
Lys-45	124	175	51	29
Pro-46	87	125	38	30
Gln-47	111	176	65	37

^a Buried surface area is calculated as the difference in calculated accessibility of residues in the isolated peptide and the peptide in the intact protein (i.e. accessible surface in isolated peptide – accessible surface area in protein).

^b Percent buried is calculated as (accessible surface in isolated peptide – accessible surface area in protein)/(accessible surface area in protein).

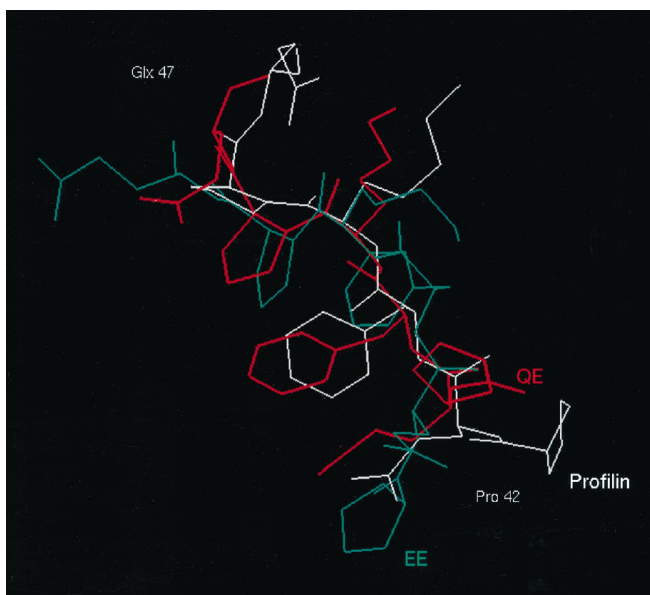


FIG. 6. **Stereo view of the alignment of the QE (birch, red) and EE (timothy, green) peptide NMR structures with the corresponding loop from the birch profilin crystal structure (white).** The C', C α , and N backbone atoms of the peptides and profilin were used in the alignment.

Gln-47 are positioned at the surface of the folded molecule and are thus accessible to the solvent. Fig. 6 shows the superposition of the two peptide epitope structures as determined by NMR with the observed crystal structure for residues 42–47 from birch pollen profilin. Although the fits were not complete (root mean sequence of 1.38 and 1.6 Å on backbone atoms of the birch and timothy peptide, respectively), the extended conformation of both peptides suggested that they may readily conform to the appropriate conformation required for antibody binding.

Based on the assumption that the free birch peptide and the epitope within the native molecule make the same contacts with mAb 4A6, two models of interaction were considered. One possibility was that 4A6 binds to the epitope without requiring a significant change in the epitope conformation. In this model, Gln-43, Lys-45, Pro-46, and Gln-47 would make extensive contacts with the CDRs, whereas Pro-42 and Phe-44 would not contact the CDRs. A second model involves a conformational change of the epitope upon binding to 4A6 such that residues with low accessibility in the native protein would make significant contributions to the binding interface. However, if Phe-44 was involved in complex formation, it would have to leave the

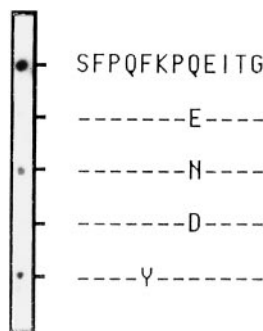


FIG. 7. Reactivity of mAb 4A6 with mutant birch profilin peptides. mAb 4A6 was tested with dot-blotted mutant peptides. The sequences and order of the peptides are displayed.

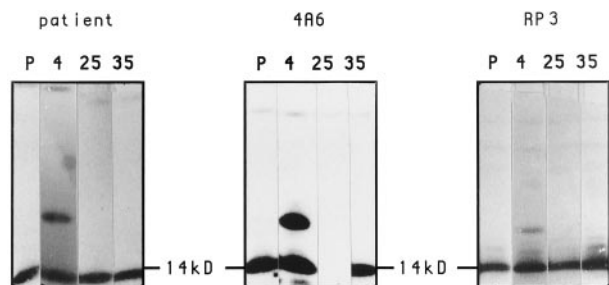


FIG. 8. Reactivity of nitrocellulose-blotted recombinant birch profilin (wild type) and birch profilin mutants with antibodies. *E. coli* extracts containing approximately 1 μ g/cm gel recombinant birch profilin wild type (P) and mutagenized birch profilins (clone 4, Phe-44 $\rightarrow$ Tyr; clone 25, Gln-47 $\rightarrow$ Glu; clone 35, Gln-47 $\rightarrow$ Asn) were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose. Nitrocelluloses were probed with serum IgE from a profilin-allergic patient, mAb 4A6, and a rabbit anti-birch carboxyl terminus antiserum.

hydrophobic core of the protein. Considering the energetic cost of such a rearrangement, this possibility seemed unlikely.

The comparison of the NMR structure of the birch peptide with the timothy peptide containing the Gln-47 $\rightarrow$ Glu exchange showed that both peptides have an extended conformation that could be superimposed to the 4A6 epitope deduced from the crystal structure of birch profilin. These data indicated that the lack of cross-reactivity of mAb 4A6 with other plant profilins was not due to a local conformational change of this epitope due to the Gln-47 $\rightarrow$ Glu exchange. Binding tests with mutant peptides (Fig. 7) demonstrated that a change of Phe-44 to Tyr-44 did not affect the 4A6 binding. Gln-47 could be exchanged to Asn-47 without altering 4A6 reactivity. However, the substitution of Gln-47 by acidic amino acids such as Glu-47 or Asp-47 abolished binding of 4A6 completely.

The peptide binding data could be reproduced using recombinant birch profilin mutants (Fig. 8). Birch profilin mutants Phe-44 $\rightarrow$ Tyr and Gln-47 $\rightarrow$ Asn were bound by mAb 4A6, whereas the Gln-47 $\rightarrow$ Glu mutant was not recognized. A band of approximately 28 kDa observed in the Phe-44 $\rightarrow$ Tyr mutant preparation was recognized by the antibodies and therefore most likely represented a dimer. Antibodies with specificity for other epitopes (serum IgE from a birch profilin allergic individual or a rabbit antiserum raised against the birch profilin carboxyl terminus RP3) reacted with all three mutant proteins. In addition all birch profilin mutants could be purified by poly-L-proline affinity chromatography, indicating correct folding and functional activity of the molecules (Vrtala *et al.*, 1996).

The binding experiments with peptides and birch profilin mutants together supported the first model of interaction, which involves Gln-43, Lys-45, Pro-46, and Gln-47 as direct contact sites of birch profilin with mAb 4A6. It is further

assumed that changes of Gln-47 to structurally similar acidic amino acids such as Glu or Asp abolished binding, most likely as a consequence of electrostatic repulsion caused by acidic amino acid residues present in the CDRs of mAb 4A6. This hypothesis was corroborated by the fact that changes of Gln-47 to Asn, an amino acid of similar structure and functionality, did not abolish binding of mAb 4A6.

In conclusion we have characterized a monoclonal antibody specific for a potent allergen that is an important component of the plant cytoskeleton. A continuous hexapeptide motif was identified as the minimal epitope and studied at the molecular and structural level. It was demonstrated that the natural immune response toward protein antigens can result in the production of peptide-directed antibodies that derive substantial binding energy from linear epitopes. These analyses may contribute to the general concepts on epitope-paratope interactions.

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