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 epitope 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 phyta that bind to actin and to polyphosphoinositols 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; Björkergren 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

* 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.

This paper is available online at http://www-jbc.stanford.edu/jbc/

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(Nicotiana tabacum), mugwort (Artemisia vulgaris), timothy grass (Phleum pratense), Zea maize, Artemisia vulgaris, and wheat (Triticum aestivum). Each sample was 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 lyophylized. Lyophylized 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 dia lyzed 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 Mi moclonal 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 (Gefter et al., 1979). The hybridoma clone producing mAb 4A6 was cultured in RPMI1640 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 peptide derived from the x-ray crystal structure of birch profilin (Valenta et al., 1993), which was used as a keyhole limpet hemocyanin (RP3) (Vallier et al., 1986). cDNA fragments were amplified by PCR using a RNA-PCR kit (Perkin Elmer). Plasmids pMW175prof, pMW175profY44, pMW175profE47, and pMW175profN47 were then transformed into E. coli K12 CAG7484 thi leu lacYonAsupB44 lacX90 dna259 thr::Tn10 (Straus et al., 1988), which was cotransformed with plasmid pT7FOL22 (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 and the homologous peptides from maize, timothy grass, tobacco, and wheat, the Boltzmann device was used (Sipil, 1980). The overall structures were then built from the ensembles as described (Sipil 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 trimethylsilyle propane. 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 t1 increments of 16 scans each. TOCSY spectra (Bax and Davis, 1985) with a 70-ms mixing time were collected as 512 t1 increments of 8 scans each, and ROESY spectra (Griesinger and Ernst, 1987) with a 300-ms mixing time were collected as 512 t1 increments of 32 scans each. Quadrature detection in t1 was achieved by the time proportional phase incrementation method (Marion and Wuthrich, 1993). 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 (Wuthrich, 1986). Conformations of the birch and timothy grass peptide were calculated by distance geometry with the program DIANA (Guntert et al., 1991), using a total of 16 residue rotating frame Overhauser effect distance restraints for the birch peptide and 56 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.
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 is 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 (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.

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 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.
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.

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 (SFPQFKPEEITG) 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, 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.
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 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
poly-L-proline affinity chromatography, indicating correct fold-

In addition all birch profilin mutants could be purified by

vidual or a rabbit antiserum raised against the birch profilin

most likely represented a dimer. Antibodies with specificity for

preparation was recognized by the antibodies and therefore

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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 substan-
tial binding energy from linear epitopes. These analyses may

contribute to the general concepts on epitope-paratope

interactions.

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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 µg/cm gel recombinant birch profilin wild type (P) and mutagenized birch profilins (clone 4, Phe-44–→ Tyr; clone 25, Gln-47–→ Glu; clone 35, Gln-47–→ Asn) were separated by SDS-polyacylamide 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.
