



Mimotopes identify conformational epitopes on parvalbumin, the major fish allergen

Eva Untersmayr^{a,1}, Krisztina Szalai^{a,1}, Angelika B. Riemer^a, Wolfgang Hemmer^b, Ines Swoboda^c, Brigitte Hantusch^d, Isabella Schöll^a, Susanne Spitzauer^c, Otto Scheiner^a, Reinhart Jarisch^b, George Boltz-Nitulescu^a, Erika Jensen-Jarolim^{a,*}

^a Center of Physiology and Pathophysiology, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

^b Floridsdorf Allergy Center (FAZ), Franz-Jonas-Platz 8/6, 1210 Vienna, Austria

^c Department of Clinical Chemistry and Laboratory Medicine, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

^d Clinical Institute of Pathology, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

Received 22 June 2005

Abstract

Parvalbumin, the major fish allergen, is recognized by allergen-specific IgE of more than 90% of all fish-allergic patients. A detailed knowledge of allergenic structures is crucial for developing a vaccine inducing blocking antibodies specifically directed towards the IgE binding epitopes. In the present study we aimed to use the phage display technique to generate mimotopes, which mimic epitopes on parvalbumin.

Parvalbumin-specific IgE was purified from sera of fish-allergic patients and used for screening of a constrained decamer phage library. After four rounds of biopanning using parvalbumin-specific IgE, five phage clones were selected which were specifically recognized by parvalbumin-specific IgE as well as IgG. DNA sequencing and peptide alignment revealed a high degree of sequence similarities between the mimotopes. Interestingly, on the surface of natural parvalbumin three regions could be defined by computational mimotope matching. In accordance, previously defined allergenic peptides of cod parvalbumin highlighted areas in close proximity or overlapping with the mimotope matching sites.

From the presented data we conclude that our approach identified conformational epitopes of parvalbumin relevant for IgE and IgG binding. We suggest that these mimotopes are suitable candidates for an epitope-specific immunotherapy of fish-allergic patients.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: Parvalbumin; Fish allergy; Mimotope; Epitopes; IgE; IgG; Computational modeling; Phage display

1. Introduction

Parvalbumins are high-affinity $\text{Ca}^{2+}/\text{Mg}^{2+}$ -binding proteins, which are found at high amounts in white muscles of low vertebrates (Berchtold, 1996) and in lower concen-

trations in skeletal muscles of higher vertebrates (Lehky et al., 1974). They are proposed to have an implication in the relaxation process of muscles, as parvalbumins buffer Ca^{2+} and transport Ca^{2+} from myofibrils to the sarcoplasmic reticulum (Berchtold et al., 2000; Heizmann et al., 1982). These proteins are uniformly of low molecular weight (12 kDa), highly soluble in water and have an isoelectric point between 4 and 5.5 (Coffee and Bradshaw, 1973; Declercq et al., 1991; Hamoir and Konosu, 1965; Konosu et al., 1965; Pechere, 1968).

In 1973, the Ca^{2+} binding site, a so-called EF-hand motif, was first discovered in the crystal structure of parvalbumins. It consists of two 8- to 10-residue α -helices and a

Abbreviations: LB, luria-bertani; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Tween 20

* Corresponding author. Tel.: +43 1 40400 5103; fax: +43 1 40400 5130.

E-mail address: erika.jensen-jarolim@meduniwien.ac.at (E. Jensen-Jarolim).

URL: <http://www.allergology.at>.

¹ These authors contributed equally to this work.

12-residue interhelical loop, forming a single Ca^{2+} binding site (Kretsinger and Nockolds, 1973). Only two of the three EF-hand motifs of parvalbumin are functional for chelating Ca^{2+} and Mg^{2+} , whereas the N-terminal AB-domain contributes to the hydrophobic core of the protein by forming a cap (Declercq et al., 1991; Kawasaki and Kretsinger, 1994).

Despite the similar features of parvalbumins from different species, these proteins have been recognized as allergens only in fish and frog (Hilger et al., 2002; O'Neil et al., 1993). Fish parvalbumin belongs to the group of major allergens as IgE from over 90% of all fish-allergic patients react with this antigen (Bugajska-Schretter et al., 1998; O'Neil et al., 1993), even though it is not the predominant protein in fish (Elsayed and Bennich, 1975).

Gad c 1, the codfish parvalbumin, has been a focus of research projects since the late 1960s, when first studies on allergenicity of codfish proteins have been performed (Aas, 1967; Aas and Jebsen, 1967). To identify IgE binding sites, peptides of Gad c 1 have been generated by tryptic hydrolysis and evaluated for IgE binding or RAST inhibition activity (Elsayed et al., 1976). Due to the high degree of amino acid homology in the identified sites, presence of multiple allergenic determinants was suggested and the Ca^{2+} binding loops of the EF-hands were postulated as IgE binding domains (Apold and Elsayed, 1980; Elsayed and Apold, 1983; Elsayed et al., 1981). However, a possible conformational nature of epitopes on parvalbumins was not addressed by these studies, even though conformation seems to be an overall precondition for the sensitization capacity of this molecule (Untersmayr et al., 2003).

In recent years, the phage display technique has become a useful tool to define peptides that mimic natural, even conformational antibody-binding epitopes on protein antigens, such as allergens, toxins and tumors (Felici et al., 1993; Förster-Waldl et al., 2005; Ganglberger et al., 2000; Hantusch et al., 2004; Riemer et al., 2005a, 2004). From random peptide phage libraries with a huge repertoire of possible ligands, antibody-specific peptides are selected by biopanning (Folgori et al., 1994). After sequencing these affinity-selected peptides, alignments and surface matching with the antigen can be performed providing information about epitope localization and the three-dimensional structure of the antibody binding-sites.

The aim of the present study was to generate mimotopes of parvalbumin for characterizing IgE epitopes of this important and highly cross-reactive fish allergen (Bugajska-Schretter et al., 1998). Thereby, we intended to take into consideration, for the first time, the structural features of this protein for epitope studies.

2. Materials and methods

2.1. Affinity purification of parvalbumin-specific IgE

Parvalbumin-specific antibodies were purified from serum samples of 16 fish-allergic patients by means of affinity

chromatography (Jensen-Jarolim et al., 1992) using rCyp c 1.01 (Swoboda et al., 2002) coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). After extensive washing with phosphate-buffered saline (PBS), specifically bound antibodies were eluted from the column with 3 M KSCN, pH 7.2. This fraction thus contained parvalbumin-specific IgE and IgG antibodies. In a second step, IgG and IgE antibodies were separated by a protein G column. The quality of the antibody fractions was checked in ELISA, sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting experiments as previously described (Schöll et al., 2002) using recombinant parvalbumin and the control proteins Bet v 1 (Biomay, Vienna, Austria), bovine serum albumin (BSA) and troponin (Sigma-Aldrich, St. Louis, MO). Moreover, fish-specific IgE levels were determined in the UniCAP System (Pharmacia).

2.2. Biopanning of a phage display library

Ligands were selected from a constrained random peptide phage library (CL10), which expresses cysteine-flanked decapeptides fused to pIII of the filamentous phage M13. The library displaying a diversity of 1.04×10^9 different peptides was a kind gift of Prof. Dr. L. Mazzucchelli (Locarno, Switzerland). Four successive rounds of biopanning with parvalbumin-specific IgE antibodies were performed with antibodies coated in 0.1 M NaHCO_3 , pH 8.5 on ELISA plates (Nunc, Roskilde, Denmark) overnight at 4 °C (1st round 0.05 µg/mL, 2nd round 0.05 µg/mL, 3rd round 0.025 µg/mL, 4th round 0.025 µg/mL) as described (Parmley and Smith, 1988). Wells were blocked with PBS/1% dry milk powder and then incubated with the library or with amplified phages from the preceding biopanning round for 2 h at room temperature. Bound phages were eluted with 0.1 M glycine buffer, pH 2.2, and immediately neutralized with 1 M Tris–HCl, pH 8.0. Phages were amplified in *Escherichia coli* XL-1 blue, and either used for the next biopanning round or stored at –80 °C. Aliquots of the eluates or the amplicates were plated in serial dilutions on luria-bertani (LB) plates containing 50 µg/mL kanamycin and 12.5 µg/mL tetracycline for titer determination.

2.3. Colony screening assay and specificity tests

After four rounds of selection, the enrichment of parvalbumin-specific phage particles in each round was verified by a capture ELISA. In short, parvalbumin-specific antibodies and human monoclonal IgE (SUS11-IgE purified from a hybridoma supernatant, (Zürcher et al., 1995) a kind gift from Prof. Dr. B.M. Stadler, Bern Switzerland) were coated (0.05 µg/mL). After blocking of unspecific binding sites with phosphate-buffered saline with Tween 20 (PBST)/1% BSA, wells were incubated with amplified phages from each round diluted 1:10 in PBST/0.1% BSA (phage concentration range between 1×10^8 and 4×10^9 cfu/mL) or buffer overnight at

4 °C. After intensive washing, bound phages were detected with a peroxidase-labeled mouse anti-phage antibody (Amersham Pharmacia Biotech, Little Chalfont, UK) and the reaction was developed with ABTS (Sigma-Aldrich, St. Louis, MO) as substrate. Subsequently, 75 clones of rounds 3 and 4 were chosen, amplified, adjusted to 1×10^9 cfu/mL and tested for reactivity with parvalbumin-specific antibodies as described earlier (Hantusch et al., 2004). Phage clones with OD values greater than the mean ± 3 S.D. of the background value were scored as positive and amplified. To test the selected clones for IgE and IgG reactivity, phages were coated onto ELISA plates at adjusted concentrations (1×10^9 cfu/mL), incubated with parvalbumin-specific IgE or IgG and bound antibodies were detected with peroxidase-labeled anti-human IgE (Kirkegaard & Perry Laboratories, Gaithersburg, MD) or anti-human IgG (Jackson ImmunoResearch, West Grove, PA), respectively.

2.4. Phage sequence analysis and peptide alignment

Phage DNA was prepared from overnight cultures of single phage clones. Single strand DNA was purified using a QiaPrep Spin M13 Kit (Qiagen, Hilden, Germany). The amount and quality of prepared DNA were checked in a 1.2% agarose gel containing 0.01% EtBr₂ in TBE buffer, visualized under UV illumination. DNA sequencing was performed using a thermo sequenase cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) with the fluorescence-labeled primer 5'-gtaccgtaacactgagtttcg-3' (MWG Biotechnik, Ebersberg, Germany) and analyzed by a LI-COR 4000L DNA sequencer (LI-COR Inc., Lincoln, NE). Sequence comparison of peptides was done by using the AlignX module of the Vector NTI Suite software (InforMax Inc., Frederick, MD), which uses a modified Clustal W algorithm (Thompson et al., 1994) to create multiple sequence alignments.

2.5. Epitope matching

Obtained mimotope primary sequences were subjected to a surface matching algorithm, a method of epitope definition (Riemer et al., 2005b). In short, the localization of epitope regions on the parvalbumin surface was achieved using mimotope amino acid sequence information and molecule spatial structure of natural carp parvalbumin (Kumar et al., 1990) as given in the RCSB Protein Data Bank accession no. 4CPV. The surface area localization scheme was a $M \times N$ variable quadratic program, where M and N were the numbers of mimotope and antigen atoms, respectively, each variable representing the distance between one mimotope atom and one antigen atom in a notional superimposition of the two molecules. The objective function, to be minimized, measured how close mimotope atoms were aligned to antigen atoms, with special consideration given to charged atoms. Distances between antigen atoms and distances between

mimotope side chain atoms were used to define the initial set of constraints. This quadratic program was solved repeatedly, each time with additional constraints harvested from previous runs having been added, until the closest superimposition not violating mimotope bond length and bond angle requirements was identified.

Furthermore, calculated matching sites were verified by applying the algorithm described by Halperin et al. (2003).

3. Results

3.1. Generation of peptides reactive with parvalbumin-specific IgE

To select for peptide ligands of polyclonal IgE specifically directed to parvalbumin, antibodies were purified from a serum pool of fish-allergic patients by affinity chromatography using recombinant parvalbumin. After separation into IgE and IgG fractions, the antibodies were analyzed for specificity in immunoblot experiments. Prominent bands at 12 kDa corresponding to the molecular weight of parvalbumin and at

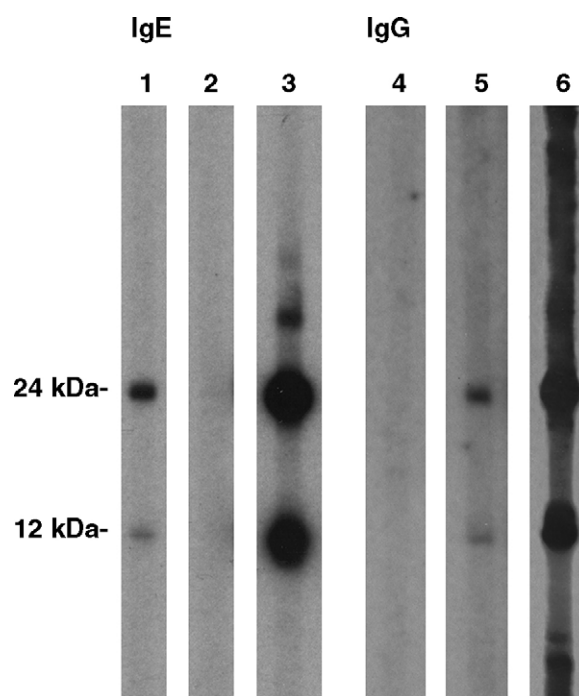


Fig. 1. Purified IgE and IgG antibodies bind specifically to recombinant parvalbumin. Antibody fractions and a serum pool of 16 fish-allergic patients were tested for parvalbumin-specific IgE or IgG in immunoblot experiments using ¹²⁵I radio-labeled anti-human IgE (lanes 1–3) and anti-human IgG (lanes 4–6) for detection. The IgE fraction contained IgE antibodies specifically binding to a 12 and 24 kDa protein (lane 1), whereas no IgG reactivity could be detected (lane 4). In the IgG fraction no IgE reactivity with parvalbumin was observed (lane 2), but binding to the 12 and 24 kDa protein was observed when screening for IgG (lane 5). Lanes 3 and 6 show IgE and IgG reactivities of the serum pool, respectively.

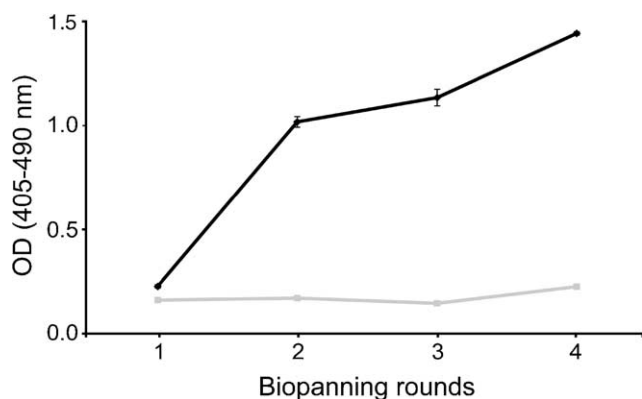


Fig. 2. Enrichment of phage particles displaying parvalbumin-specific peptides during biopanning. A cyclic decamer random peptide phage library was used for biopanning with parvalbumin-specific antibodies purified from fish-allergic patients. Four successive biopanning rounds were performed. The increase of specific phage titers was determined by capture ELISA using purified parvalbumin-specific IgE (black line) or control hybridoma IgE (grey line) for catching. Bound phage particles were detected by anti-phage antibodies and the mean of measurements $\pm$ S.D. are shown.

the double size were recognized by both, IgE and IgG (Fig. 1, lanes 1 and 5). The same binding patterns were observed when testing with the serum pool of 16 fish-allergic patients (Fig. 1, lanes 3 and 6). Furthermore, in this experiment the separation of purified IgE and IgG antibodies was demonstrated as no IgG could be detected in the IgE fraction and vice versa (Fig. 1, lanes 2 and 4). Moreover, the specificity of our antibody fractions was confirmed as no binding activity with the control antigens Bet v 1, BSA or Troponin was observed (data not shown). The concentration of fish-specific IgE antibodies in the IgE fraction was determined to be 523.2 ng/mL in UniCAP.

The purified anti-parvalbumin IgE antibodies were applied for biopanning of a constrained decamer random peptide phage library. As a first indicator of successful biopanning, an increase of phage titers recognized by parvalbumin-specific antibodies was observed after the four rounds of selection (Fig. 2). By capture ELISA, a colony screening assay was performed to select for specific phage clones. Out of 75 tested clones, the 5 most reactive were chosen, which were specifically recognized by a fraction containing parvalbumin-specific IgE and IgG antibodies, but not by a control IgE antibody. In this experiment, a wild type control phage at adjusted phage particle concentrations was not recognized by the parvalbumin-specific antibodies (Fig. 3). To dissect between IgE and IgG binding clones, phage clones were coated onto ELISA plates and detected by parvalbumin-specific IgE or IgG fractions, respectively. Interestingly, the binding patterns of the clones observed for IgE (Fig. 4, panel A) was paralleled by the IgG reactivity (Fig. 4, panel B) indicating a specific recognition of the same peptides by both isotypes.

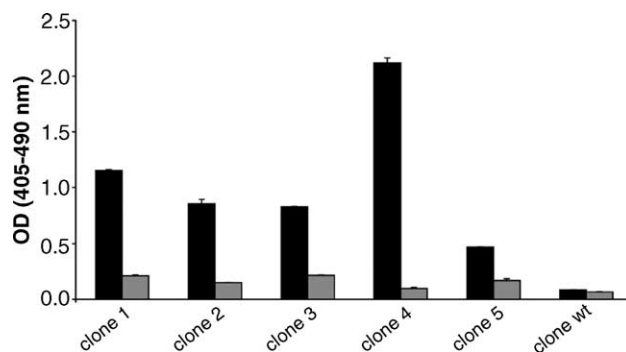


Fig. 3. Phage clones with selected peptide inserts are specifically recognized by affinity-purified parvalbumin-specific antibodies. In a catching ELISA reactivity was only observed when testing with parvalbumin-specific IgE and IgG antibodies (black columns), whereas no binding is observed using SUS11-IgE for control (grey columns). Furthermore, no reactivity was observed with a wild type control phage. Bound phage particles were detected by anti-phage antibodies and the mean of measurements $\pm$ S.D. are shown.

3.2. Sequence analysis and alignment of selected peptides

The five selected clones were amplified and their DNA was sequenced. Even though all five phage clones rendered different peptide sequences, a high degree of amino acid similarities was observed. As depicted in Fig. 5, identical and related amino acid residues were identified within the peptides. The degree of similarity is illustrated by a phylogenetic tree indicating the highest relationship between mimotopes C-YRGVTLAGHR-C, C-FKGVRLDGTP-C and C-YRGARVDGLM-C.

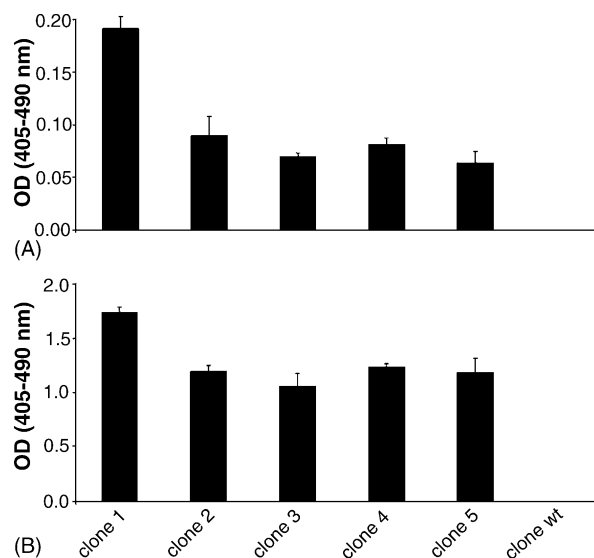


Fig. 4. Selected phage clones bind parvalbumin-specific IgE as well as IgG. (A) Coated phage clones (1–5) were specifically recognized by anti-parvalbumin IgE antibodies. (B) The IgE reactivity pattern was paralleled by recognition of clones through parvalbumin-specific IgG. Reactivity of antibodies with wild type control phages was subtracted and the results are presented as mean $\pm$ S.D.

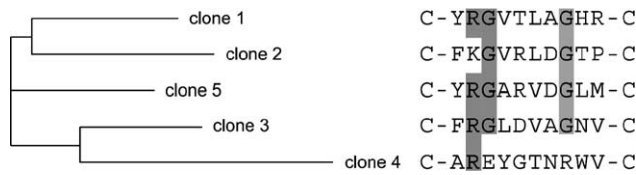


Fig. 5. Phylogenetic tree illustrating the sequence similarities between selected peptides. The alignment of peptide sequences revealed a high degree of sequence homology between the selected clones. Identical amino acid residues were boxed in dark grey, related residues were highlighted in light grey.

3.3. Localization of epitopes on parvalbumin

Based on structural data of parvalbumin, computational matching of mimotopes onto the solvent-exposed surface of natural carp parvalbumin was performed. Three major epitope areas on the molecule were revealed (Fig. 6, panel A), which were confirmed by two independent matching algorithms. (1) Mimotopes C-YRGVTLAGHR-C and C-YRGARVDGLM-C matched to aa 23 and 25–29. Mimotope C-FKGVRLDGTP-C matched to aa 33–37. Due to their close proximity, the two matches were combined to one area as indicated in light grey/green in Fig. 6. (2) Mimotope C-AREYGTNRWV-C matched to aa 77–79 (Fig. 6, panel A, highlighted in dark gray/magenta). (3) The mimotope C-FRGLDVAGNV-C matched to aa 87, 89–92, 94 (Fig. 6, panel A, shown in black/red). When comparing these sites on the molecule with the location of previously defined allergenic peptides of cod parvalbumin (Fig. 6, panel B), overlapping areas or a close proximity of the epitopes were observed.

4. Discussion

During recent years, major research efforts have been made to characterize epitopes on food allergens for the development of novel diagnostic tools and for specific therapy. As immunotherapy with food allergen extracts is not feasible due to the high anaphylactic risk (Oppenheimer et al., 1992), knowledge about the IgE reactive sites is crucial for the design of safe therapeutics. In numerous studies synthetic peptides of various allergen sources have been generated and identified as IgE-reactive epitopes using patients' sera (Ayuso et al., 2002; Battais et al., 2005; Glaspole et al., 2005; Jarvinen et al., 2002; Shimojo et al., 1994). Also for Gad c 1, the parvalbumin of codfish, peptides have been generated by tryptic hydrolysis and analysed for IgE binding (Apold and Elsayed, 1980; Elsayed and Apold, 1983; Elsayed et al., 1981). However, as most antibody epitopes are described to be conformational (Aalberse, 2000), peptides identified by sequential approaches may render limited information and, moreover, might exhibit reduced antibody-binding affinity. For these reasons we aimed to use the phage display technique to generate peptide mimics allowing the characterization of

conformational epitopes of the major fish allergen parvalbumin.

One aim of this study was to definitely answer the question whether IgE epitopes could be identical to IgG binding sites. Therefore, biopanning was performed with highly purified IgE specific for parvalbumin. This procedure enabled us to specifically select for clones mimicking IgE binding sites on parvalbumin. Despite the very low antibody concentration in the IgE fraction, five different clones could be identified. Interestingly, all five phage-displayed peptides showed similar binding patterns with IgE as well as with IgG in ELISA experiments, indicating that we identified epitope mimics relevant for both isotypes. This may indicate an IgE production via a sequential class switch in the patients (Aalberse and Platts-Mills, 2004) resulting in formation of both, IgE and IgG antibodies with the same epitope specificity. However, as the mimotopes were selected by IgE antibodies, additional IgG epitopes on parvalbumin cannot be ruled out.

Alignments among the five selected mimotopes revealed a remarkable degree of similarity between the amino acid sequences. Interestingly, by computational matching of the mimotopes onto the molecular surface of natural parvalbumin three epitope regions were identified. Two of them are located on the axis joining the AB and CD domain and the CD and EF-hand, respectively, whereas the third matching region is part of the EF domain of parvalbumin. Moreover, comparing the localizations of previously described allergenic peptides of cod parvalbumin (Elsayed and Apold, 1983) and our mimotope matches using the three-dimensional model of carp parvalbumin revealed overlapping areas or adjacent regions as antibody-binding sites. Due to the high degree of identical amino acids between carp and cod parvalbumin (64.6%) the concordance was accepted as an indicator of the importance of these regions as inter-species cross-reactive epitopes. Especially the third matching site in the EF domain area has an identical sequence in both molecules, strengthening the relevance of this area as an epitope region. It has been shown previously that Ca^{2+} depletion results in a reduced IgE binding capacity of parvalbumin (Bugajska-Schretter et al., 1998), which may be ascribed to dramatic conformational changes induced upon Ca^{2+} binding (Declercq et al., 1991; Ikura, 1996; Trave et al., 1995). As the Ca^{2+} binding loop of the EF domain was confirmed to be an immunogenic area in the present study, the conformational changes might result in inaccessibility of the residues involved in antibody binding. However, taking into consideration the numbers of residues involved in B-cell epitopes (Laver et al., 1990), all three regions in concert may be involved in the antigen–antibody interaction.

In conclusion, we describe here IgE epitopes on the major fish allergen parvalbumin taking for the first time into consideration the conformational structure of the intact protein. The knowledge of IgE epitopes on parvalbumin may in the future facilitate epitope-specific vaccination strategies in the treatment of fish allergy. It was shown in previous studies

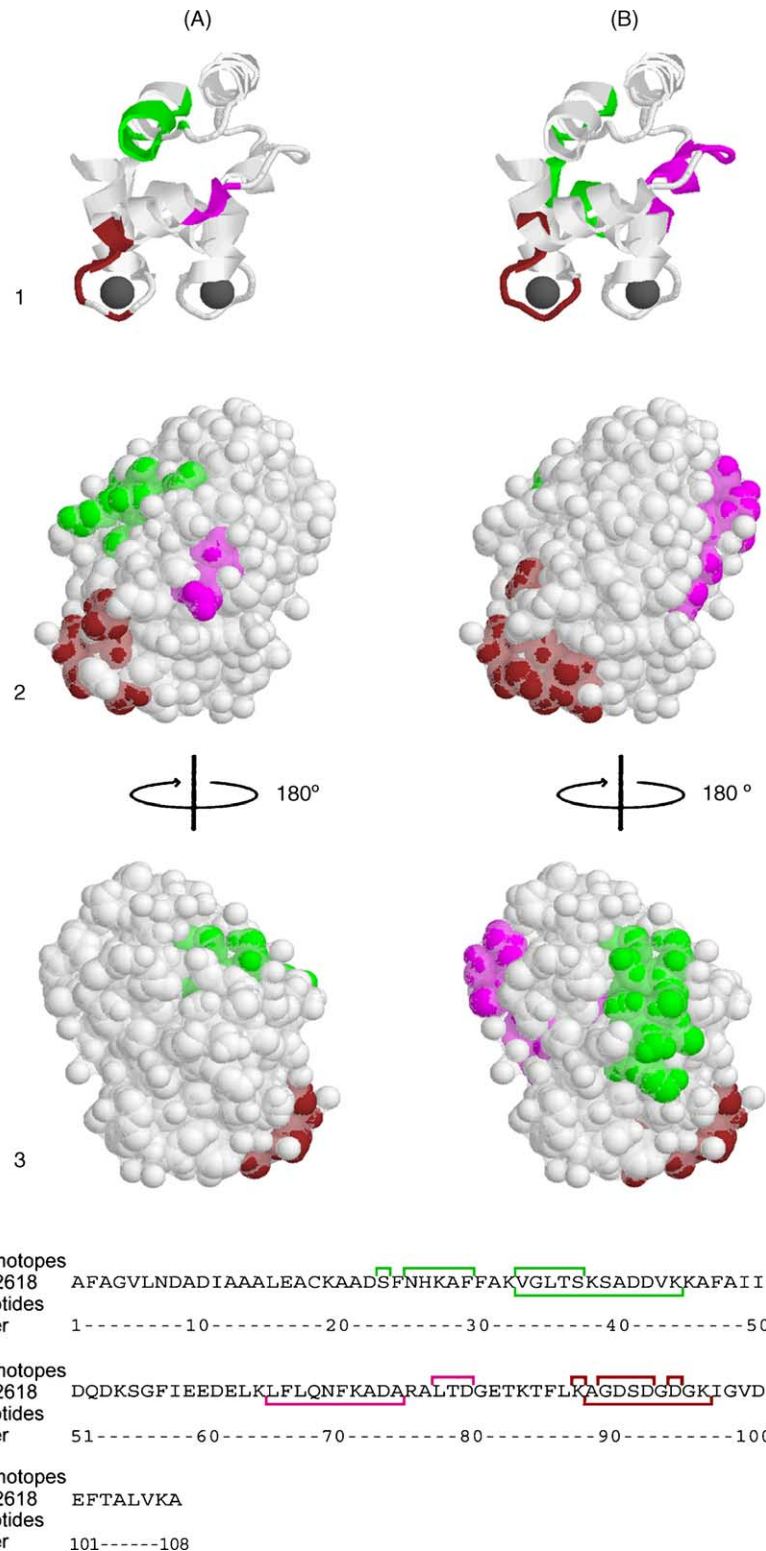


Fig. 6. Computational matching of mimotopes to the solvent-exposed amino acids of natural carp parvalbumin. (A) Matching areas of selected mimotopes are shown: aa 23, 25–29 (corresponding to mimotopes 1 and 5) and 33–37 (mimotope 2) are highlighted in light grey/green, aa 77–79 (mimotope 4) in dark grey/magenta and aa 87, 89–92, 94 (mimotope 3) in black/red. (B) Gad c 1 peptides, which were previously characterized as IgE binders, are highlighted on the surface of carp parvalbumin. (1) Ribbon diagram of the calcium-loaded parvalbumin molecule. (2) Molecular surface presentation. (3) 180° Rotation of the molecule. In the diagram below, the localisation of the mimotope matches (above sequence) and of the peptides (below sequence) are indicated by brackets on the primary sequence of natural carp parvalbumin. This figure was created with the program Protein Explorer 2.411 Beta®.

that immunizations with mimotope peptides indeed induce antigen-specific antibodies (Hantusch et al., 2004; Jensen-Jarolim et al., 1998; Riemer et al., 2005a; Schöll et al., 2002). Moreover, by an epitope-specific approach anaphylactogenic IgG specificities can be excluded (Jensen-Jarolim et al., 1999) in favor of directed induction of blocking IgG (Ganglberger et al., 2000). Thus, the here described IgE reactive mimotopes represent promising vaccination tools, especially as their effectiveness may be validated in our murine fish allergy model (Untersmayr et al., 2004, 2003).

Acknowledgements

We would like to thank Ing. Magdolna Vermes and Harald Kurz for excellent technical assistance. This study was supported by grant 10326 of the Austrian National bank “Jubiläumsfond” and by grant SFB- F1808-B04 of the Austrian National Science Foundation (FWF). Ines Swoboda and Susanne Spitzauer were supported by grant SFB- F1804-B04 of the FWF.

References

- Aalberse, R.C., 2000. Structural biology of allergens. *J. Allergy Clin. Immunol.* 106, 228–238.
- Aalberse, R.C., Platts-Mills, T.A.E., 2004. How do we avoid developing allergy: modifications of the TH2 response from a B-cell perspective. *J. Allergy Clin. Immunol.* 113, 983–986.
- Aas, K., 1967. Studies of hypersensitivity to fish. Studies of different fractions of extracts from cod muscle tissue. *Int. Arch. Allergy Appl. Immunol.* 31, 239–260.
- Aas, K., Jebsen, J.W., 1967. Studies of hypersensitivity to fish. Partial purification and crystallization of a major allergenic component of cod. *Int. Arch. Allergy Appl. Immunol.* 32, 1–20.
- Apold, J., Elsayed, S., 1980. The immunochemical reactivity of regions encompassing Tyr-30 and Arg-75 of allergen M from cod. *Mol. Immunol.* 17, 291–306.
- Ayuso, R., Lehrer, S.B., Reese, G., 2002. Identification of continuous, allergenic regions of the major shrimp allergen Pen a 1 (tropomyosin). *Int. Arch. Allergy Immunol.* 127, 27–37.
- Battais, F., Mothes, T., Moneret-Vautrin, D.A., Pineau, F., Kanny, G., Popineau, Y., Bodinier, M., Denery-Papini, S., 2005. Identification of IgE-binding epitopes on gliadins for patients with food allergy to wheat. *Allergy* 60, 815–821.
- Berchtold, M.W., 1996. Parvalbumin. In: Celio, M.R. (Ed.), *Guidebook to the Calcium-Binding Proteins*. Oxford University Press, Oxford, UK, pp. 123–126.
- Berchtold, M.W., Brinkmeier, H., Muntener, M., 2000. Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. *Physiol. Rev.* 80, 1215–1265.
- Bugajska-Schretter, A., Elfman, L., Fuchs, T., Kapiotis, S., Rumpold, H., Valenta, R., Spitzauer, S., 1998. Parvalbumin, a cross-reactive fish allergen, contains IgE-binding epitopes sensitive to periodate treatment and Ca^{2+} depletion. *J. Allergy Clin. Immunol.* 101, 67–74.
- Coffee, C.J., Bradshaw, R.A., 1973. Carp muscle calcium-binding protein. I. characterization of the tryptic peptides and the complete amino acid sequence of component B. *J. Biol. Chem.* 248, 3302–3312.
- Declercq, J.P., Tinant, B., Parello, J., Rambaudo, J., 1991. Ionic interactions with parvalbumins. Crystal structure determination of pike 410 parvalbumin in four different ionic environments. *J. Mol. Biol.* 220, 1017–1039.
- Elsayed, S., Apold, J., 1983. Immunochemical analysis of cod fish allergen M: locations of the immunoglobulin binding sites as demonstrated by the native and synthetic peptides. *Allergy* 38, 449–459.
- Elsayed, S., Apold, J., Aas, K., Bennich, H., 1976. The allergenic structure of allergen M from cod I. Tryptic peptides of fragment TM 1. *Int. Arch. Allergy Appl. Immunol.* 52, 59–63.
- Elsayed, S., Bennich, H., 1975. The primary structure of allergen M from cod. *Scand. J. Immunol.* 4, 203–208.
- Elsayed, S., Ragnarsson, U., Apold, J., Florvaag, E., Vik, H., 1981. Allergenic synthetic peptide corresponding to the second calcium-binding loop of cod allergen M. *Scand. J. Immunol.* 14, 207–211.
- Felici, F., Luzzago, A., Folgori, A., Cortese, R., 1993. Mimicking of discontinuous epitopes by phage-displayed peptides II. Selection of clones recognized by a protective monoclonal antibody against the Bordetella pertussis toxin from phage peptide libraries. *Gene* 128, 21–27.
- Folgori, A., Tafi, R., Meola, A., Felici, F., Galfre, G., Cortese, R., Monaci, P., Nicosia, A., 1994. A general strategy to identify mimotopes of pathological antigens using only random peptide libraries and human sera. *Embo J.* 13, 2236–2243.
- Förster-Waldl, E., Riemer, A.B., Dehof, A.K., Neumann, D., Brämswig, K., Boltz-Nitulescu, G., Pehamberger, H., Zielinski, C.C., Scheiner, O., Pollak, A., Lode, H., Jensen-Jarolim, E., 2005. Isolation and structural analysis of peptide mimotopes for the disialoganglioside GD2, a neuroblastoma tumor antigen. *Mol. Immunol.* 42, 319–325.
- Ganglberger, E., Grünberger, K., Spöner, B., Radauer, C., Breiteneder, H., Boltz-Nitulescu, G., Scheiner, O., Jensen-Jarolim, E., 2000. Allergen mimotopes for three-dimensional epitope search and induction of antibodies inhibiting human IgE. *FASEB J.* 14, 2177–2184.
- Glaspole, I.N., de Leon, M.P., Rolland, J.M., O’Hehir, R.E., 2005. Characterization of the T-cell epitopes of a major peanut allergen, Ara h 2. *Allergy* 60, 35–40.
- Halperin, I., Wolfson, H., Nussinov, R., 2003. SiteLight: binding-site prediction using phage display libraries. *Protein Sci.* 12, 1344–1359.
- Hamoir, G., Konosu, S., 1965. Carp myosins of white and red muscles. General composition and isolation of low-molecular-weight components of abnormal amino acid composition. *Biochem. J.* 96, 85–97.
- Hantusch, B., Krieger, S., Untersmayr, E., Schöll, I., Knittelfelder, R., Flicker, S., Spitzauer, S., Valenta, R., Boltz-Nitulescu, G., Scheiner, O., Jensen-Jarolim, E., 2004. Mapping of conformational IgE epitopes on Phl p 5a by using mimotopes from a phage display library. *J. Allergy Clin. Immunol.* 114, 1294–1300.
- Heizmann, C.W., Berchtold, M.W., Rowleron, A.M., 1982. Correlation of parvalbumin concentration with relaxation speed in mammalian muscles. *Proc. Natl. Acad. Sci. U.S.A.* 79, 7243–7247.
- Hilger, C., Grigioni, F., Thill, L., Mertens, L., Hentges, F., 2002. Severe IgE-mediated anaphylaxis following consumption of fried frog legs: definition of alpha-parvalbumin as the allergen in cause. *Allergy* 57, 1053–1058.
- Ikura, M., 1996. Calcium-binding and conformational response in EF-hand proteins. *Trends Biochem. Sci.* 21, 14–17.
- Jarvinen, K.M., Beyer, K., Vila, L., Chatchatee, P., Busse, P.J., Sampson, H.A., 2002. B-cell epitopes as a screening instrument for persistent cow’s milk allergy. *J. Allergy Clin. Immunol.* 110, 293–297.
- Jensen-Jarolim, E., Leitner, A., Kalchauer, H., Zürcher, A., Ganglberger, E., Bohle, B., Scheiner, O., Boltz-Nitulescu, G., Breiteneder, H., 1998. Peptide mimotopes displayed by phage inhibit antibody binding to bet v 1, the major birch pollen allergen, and induce specific IgG response in mice. *FASEB J.* 12, 1635–1642.
- Jensen-Jarolim, E., Vogel, M., de Weck, A.L., Stadler, B.M., 1992. Anti-IgE autoantibodies mistaken for specific IgG. *J. Allergy Clin. Immunol.* 89, 31–43.
- Jensen-Jarolim, E., Wiedermann, U., Ganglberger, E., Zürcher, A., Stadler, B.M., Boltz-Nitulescu, G., Scheiner, O., Breiteneder, H., 1999. Allergen mimotopes in food enhance type I allergic reactions in mice. *FASEB J.* 13, 1586–1592.

- Kawasaki, H., Kretsinger, R.H., 1994. Calcium-binding proteins. 1: EF-hands. *Protein Profile* 1, 343–517.
- Konosu, S., Hamoir, G., Pechere, J.F., 1965. Carp myogens of white and red muscles. Properties and amino acid composition of the main low-molecular-weight components of white muscle. *Biochem. J.* 96, 98–112.
- Kretsinger, R.H., Nockolds, C.E., 1973. Carp muscle calcium-binding protein. II. Structure determination and general description. *J. Biol. Chem.* 248, 3313–3326.
- Kumar, V.D., Lee, L., Edwards, B.F., 1990. Refined crystal structure of calcium-liganded carp parvalbumin 4.25 at 1.5-Å resolution. *Biochemistry* 29, 1404–1412.
- Laver, W.G., Air, G.M., Webster, R.G., Smith-Gill, S.J., 1990. Epitopes on protein antigens: misconceptions and realities. *Cell* 61, 553–556.
- Lehky, P., Blum, H.E., Stein, E.A., Fischer, E.H., 1974. Isolation and characterization of parvalbumins from the skeletal muscle of higher vertebrates. *J. Biol. Chem.* 249, 4332–4334.
- O'Neil, C., Helbling, A.A., Lehrer, S.B., 1993. Allergic reactions to fish. *Clin. Rev. Allergy* 11, 183–200.
- Oppenheimer, J.J., Nelson, H.S., Bock, S.A., Christensen, F., Leung, D.Y., 1992. Treatment of peanut allergy with rush immunotherapy. *J. Allergy Clin. Immunol.* 90, 256–262.
- Parmley, S.F., Smith, G.P., 1988. Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. *Gene* 73, 305–318.
- Pechere, J.F., 1968. Muscular parvalbumins as homologous proteins. *Comp. Biochem. Physiol.* 24, 289–295.
- Riemer, A.B., Hantusch, B., Sponer, B., Kraml, G., Hafner, C., Zielinski, C.C., Scheiner, O., Pehamberger, H., Jensen-Jarolim, E., 2005a. High-molecular-weight melanoma-associated antigen mimotope immunizations induce antibodies recognizing melanoma cells. *Cancer Immunol. Immunother.* 54, 677–684.
- Riemer, A.B., Klinger, M., Wagner, S., Bernhaus, A., Mazzucchelli, L., Pehamberger, H., Scheiner, O., Zielinski, C.C., Jensen-Jarolim, E., 2004. Generation of peptide mimics of the epitope recognized by trastuzumab on the oncogenic protein Her-2/neu. *J. Immunol.* 173, 394–401.
- Riemer, A.B., Kraml, G., Scheiner, O., Zielinski, C.C., Jensen-Jarolim, E., 2005b. Matching of trastuzumab (Herceptin (R)) epitope mimics onto the surface of Her-2/neu—a new method of epitope definition. *Mol. Immunol.* 42, 1121–1124.
- Schöll, I., Wiedermann, U., Förster-Waldl, E., Ganglberger, E., Baier, K., Boltz-Nitulescu, G., Scheiner, O., Ebner, C., Jensen-Jarolim, E., 2002. Phage-displayed Bet mim 1, a mimotope of the major birch pollen allergen Bet v 1, induces B cell responses to the natural antigen using bystander T cell help. *Clin. Exp. Allergy* 32, 1583–1588.
- Shimono, N., Katsuki, T., Coligan, J.E., Nishimura, Y., Sasazuki, T., Tsunoo, H., Sakamaki, T., Kohno, Y., Niimi, H., 1994. Identification of the disease-related T cell epitope of ovalbumin and epitope-targeted T cell inactivation in egg allergy. *Int. Arch. Allergy Immunol.* 105, 155–161.
- Swoboda, I., Bugajska-Schretter, A., Verdino, P., Keller, W., Sperr, W.R., Valent, P., Valenta, R., Spitzauer, S., 2002. Recombinant carp parvalbumin, the major cross-reactive fish allergen: a tool for diagnosis and therapy of fish allergy. *J. Immunol.* 168, 4576–4584.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Trave, G., Lacombe, P.J., Pfuhl, M., Saraste, M., Pastore, A., 1995. Molecular mechanism of the calcium-induced conformational change in the spectrin EF-hands. *EMBO J.* 14, 4922–4931.
- Untersmayr, E., Ellinger, A., Beil, W.J., Jensen-Jarolim, E., 2004. Eosinophils accumulate in the gastric mucosa of food-allergic mice. *Int. Arch. Allergy Immunol.* 135, 1–2.
- Untersmayr, E., Schöll, I., Swoboda, I., Beil, W.J., Förster-Waldl, E., Walter, F., Riemer, A., Kraml, G., Kinaciyan, T., Spitzauer, S., Boltz-Nitulescu, G., Scheiner, O., Jensen-Jarolim, E., 2003. Antacid medication inhibits digestion of dietary proteins and causes food allergy: a fish allergy model in BALB/c mice. *J. Allergy Clin. Immunol.* 112, 616–623.
- Zürcher, A.W., Lang, A.B., Aebischer, I., Miescher, S., Stadler, B.M., 1995. IgE-producing hybridomas established after B-cell culture in the CD40 system. *Immunol. Lett.* 46, 49–57.