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The Journal of
Immunology

RESEARCH ARTICLE | MAY 15 2007

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J Immunol (2007) 178 (10): 6290–6296.

<https://doi.org/10.4049/jimmunol.178.10.6290>

Related Content

Recombinant Carp Parvalbumin, the Major Cross-Reactive Fish Allergen: A Tool for Diagnosis and Therapy of Fish Allergy

J Immunol (May,2002)

A Recombinant Hypoallergenic Parvalbumin Mutant for Immunotherapy of IgE-Mediated Fish Allergy¹

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IgE-mediated allergy to fish is a frequent cause of severe anaphylactic reactions. Parvalbumin, a small calcium-binding protein, is the major fish allergen. We have recently isolated a cDNA coding for carp parvalbumin, Cyp c 1, and expressed in *Escherichia coli* a recombinant Cyp c 1 molecule, which contained most IgE epitopes of saltwater and freshwater fish. In this study, we introduced mutations into the calcium-binding domains of carp parvalbumin by site-directed mutagenesis and produced in *E. coli* three parvalbumin mutants containing amino acid exchanges either in one (single mutants; Mut-CD and Mut-EF) or in both of the calcium-binding sites (double mutant; Mut-CD/EF). Circular dichroism analyses of the purified derivatives and the wild-type allergen showed that Mut-CD/EF exhibited the greatest reduction of overall protein fold. Dot blot assays and immunoblot inhibition experiments performed with sera from 21 fish-allergic patients showed that Mut-CD/EF had a 95% reduced IgE reactivity and represented the derivative with the least allergenic activity. The latter was confirmed by in vitro basophil histamine release assays and in vivo skin prick testing. The potential applicability for immunotherapy of Mut-CD/EF was demonstrated by the fact that mouse IgG Abs could be raised by immunization with the mutated molecule, which cross-reacted with parvalbumins from various fish species and inhibited the binding of fish-allergic patients' IgE to the wild-type allergen. Using the hypoallergenic carp parvalbumin mutant Mut-CD/EF, it may be possible to treat fish allergy by immunotherapy. *The Journal of Immunology*, 2007, 178: 6290–6296.

The most severe manifestation of IgE-mediated allergy is anaphylaxis. Life-threatening anaphylaxis occurs upon systemic allergen uptake, as it may occur, for example, in insect venom, drugs, and food-allergic patients (1–3). In industrialized countries, food allergy is the most common cause of life-threatening anaphylaxis (4–6). Fish is a very frequent elicitor of IgE-mediated food hypersensitivity reactions (7–10) and represents a serious health problem especially in countries with high fish consumption (11). Based on the clinical appearance and the underlying immunological mechanisms, fish allergy belongs to the recently designated class I food allergies, where the primary sensitization process occurs via the gastrointestinal tract and anaphylactic reactions are frequent (12, 13).

Parvalbumin, a small (12 kDa) calcium-binding muscle protein with remarkable resistance to heat, denaturing chemicals, and proteolytic enzymes (14), is the major cross-reactive allergen in fish. It belongs to a family of calcium-binding proteins that is characterized by the presence of helix-loop-helix metal binding domains, termed EF-hands (15). Parvalbumins contain three such EF-hand motifs (AB, CD, and EF sites; see Fig. 1) (16–18). Two of the sites (CD and EF) are paired to form a stable domain capable of binding two cations, Ca²⁺ or Mg²⁺. The first site (AB) is unable to bind cations, but forms a cap that covers the hydrophobic surface of the pair of functional domains and thereby acts as a stabilizing element (19–21).

Recently, we expressed in *Escherichia coli* a folded recombinant parvalbumin from carp, rCyp c 1, which displayed immunological features comparable to its natural counterpart and contained the majority of IgE epitopes present in protein extracts of various fish species (22). The aim of the present study was to design hypoallergenic derivatives of rCyp c 1 with reduced allergenic activity for immunotherapy of fish allergy. Based on the observation that calcium depletion by chelating chemicals significantly reduced the IgE-binding capacity of rCyp c 1 (22), we introduced point mutations in the functional calcium-binding sites of rCyp c 1 (CD and EF sites; Fig. 1) by site-directed mutagenesis. The recombinant parvalbumin mutants were purified to homogeneity and characterized regarding their structural and immunological properties. Using in vitro IgE-binding assays and basophil histamine release tests as well as in vivo skin tests in allergic patients, we studied the allergenic activity of the mutants. A double mutant (Mut-CD/EF) containing point mutations in both calcium-binding domains exhibited a consistently reduced IgE reactivity and >50-fold reduced allergic activity. Mice were immunized with Mut-CD/EF to study whether Mut-CD/EF-specific mouse IgG Abs recognize the wild-type

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Received for publication September 1, 2006. Accepted for publication February 26, 2007.

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¹ This work was supported by Grants F01804, F01805, F01809, and F01815 from the Austrian Science Fund, a research grant from the Christian Doppler Association, and Biomay (Vienna, Austria).

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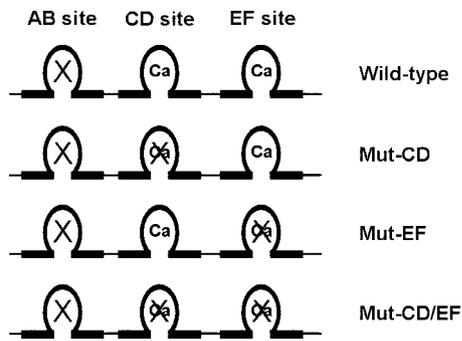


FIGURE 1. Schematic representation of the EF-hand helix-loop-helix structure of parvalbumin (Wild-type) and the three parvalbumin mutants (Mut-CD, Mut-EF, Mut-CD/EF). α -Helices are represented by black boxes, the functional Ca^{2+} -binding loops of the CD and EF sites are indicated by Ca, the abortive loop of the AB site is marked with X, and mutated loops are marked with X superimposed over Ca.

allergen and inhibit its recognition by IgE Abs from fish-allergic patients.

Materials and Methods

Characterization of fish-allergic patients

Sera were obtained from 25 patients with a positive case history of type I allergy to fish, who had experienced at least one of the typical clinical symptoms (dermatitis, urticaria, angioedema, diarrhea, rhinitis, asthma, or anaphylactic reaction) after contact with fish proteins. Diagnosis of IgE-mediated fish allergy was verified by determination of fish-specific IgE Abs using the CAP-FEIA system (Phadia). For eight patients, including the patients used for the basophil histamine release assay (patient 22) and the skin prick test (patient 25), a detailed clinical and serological characterization was available and is displayed in Table I.

Construction, *E. coli* expression, and purification of recombinant parvalbumin mutants

To modify the carp parvalbumin cDNA Cyp c 1.01 (EMBL accession no. AJ292211) in one or both of the functional calcium-binding domains (Fig. 1; CD and EF site), site-directed mutagenesis was conducted using the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene). The Ca^{2+} binding domains were mutated by replacing the first and third acidic amino acids of the calcium-binding loops (Asp) by nonpolar Ala residues. Mutagenesis experiments were performed according to the manufacturer's instructions with two synthetic oligonucleotides (mutCD and mutEF), using the Cyp c 1.01 cDNA cloned into the expression vector pET-17b (22) as a template. Both oligonucleotides encompassed 45 bp of the parvalbumin cDNA. Primer mutCD (5'-AAG GCC TTT GCT GTC ATT GCC CAA GCC AAG AGC GGC TTC ATT GAG-3') introduced changes in codons 51 (GAC \rightarrow GCC) and 53 (GAC \rightarrow GCC) and mutEF

(5'-GCC TTC CTG AAA GCT GGA GCC TCT GCT GGT GAT GGC AAG ATT GGA-3') in codons 90 (GAC \rightarrow GCC) and 92 (GAT \rightarrow GCT), respectively. Modified codons are underlined in the oligonucleotide sequences. Modifications were confirmed by dideoxynucleotide chain-termination sequencing (23) using a T7 sequencing kit (Phadia), and the resulting proteins (schematically depicted in Fig. 1) were termed Mut-CD (mutation in the CD site), Mut-EF (mutation in the EF site), and Mut-CD/EF (mutated in both calcium-binding sites).

Recombinant proteins were expressed in liquid cultures of *E. coli* BL21(DE3), which had been grown to an OD_{600} of 0.4 at 37°C (wild-type parvalbumin, Mut-CD, and Mut-EF) or to an OD_{600} of 0.15 at 26°C (Mut-CD/EF) in LB medium containing 100 mg/L ampicillin. Protein synthesis was induced by adding isopropyl β -D-thiogalactoside to a final concentration of 0.5 mM. After culturing for 4 h, cells were harvested by centrifugation, resuspended in 10 mM Tris-HCl (pH 7.5) and 1 mM PMSF, and lysed by repeated cycles of freezing in liquid nitrogen and thawing in an ice-water bath. After stirring for 60 min on ice, the bacterial cell lysates were centrifuged at $20,000 \times g$ for 30 min at 4°C and the cleared supernatants were applied to DEAE cellulose-Sepharose columns (DEAE Sepharose Fast Flow; GE Healthcare). In the case of wild-type parvalbumin, Mut-CD, and Mut-EF, fractions containing the purified proteins were eluted with a linear salt gradient (0–0.5 M NaCl in 10 mM Tris (pH 7.5)) and dialyzed against distilled water. After lyophilization the protein pellets were resuspended in distilled water. In the case of Mut-CD/EF, the flow-through fraction of the DEAE cellulose-Sepharose column, containing the recombinant protein, was first dialyzed against 100 mM sodium phosphate buffer (pH 7.5) and 2 mM 2-ME. Then, the protein was enriched in the soluble fraction by addition of 10% ammonium sulfate and removal of precipitated contaminating proteins by centrifugation ($20,000 \times g$ for 30 min at 4°C). The supernatant was applied to hydrophobic interaction chromatography using a phenyl-Sepharose column (GE Healthcare). Fractions containing purified Mut-CD/EF were eluted with a linear gradient (0.42–0 M ammonium sulfate) and dialyzed against distilled water. After lyophilization the protein pellet was resuspended in 10 mM Tris-HCl (pH 9.0).

Purity of the recombinant proteins was judged by Coomassie brilliant blue staining of 15% SDS polyacrylamide gels (SDS-PAGE) (24), and the protein concentrations were determined with a Micro BCA kit (Pierce) using BSA as a standard.

Circular dichroism analysis of purified recombinant carp parvalbumin and of the parvalbumin mutants

Circular dichroism measurements were performed in PBS on a Jasco J-715 spectropolarimeter with protein concentrations between 12.3–24.0 μM using a 1 mm path-length quartz cuvette (Hellma) equilibrated at 20°C. Spectra were recorded from 197 to 260 nm with 0.2 nm resolution at a scan speed of 50 nm/min and resulted from the average of three scans. The final spectra were corrected by subtracting the corresponding baseline spectrum obtained under identical conditions. Results were expressed as the mean residue ellipticity (Θ) at a given wavelength.

Dot blot analyses and IgE immunoblot inhibition experiments

Reactivities of recombinant carp parvalbumin and the parvalbumin mutants with serum IgE from fish-allergic patients were determined in dot blot experiments. For this purpose, 0.8 μg of aliquots of the purified proteins were dotted in duplicates on nitrocellulose membranes (Schleicher & Schuell Microscience) and were exposed to patients' sera diluted 1/10 in PBST (PBS (pH 7.5) containing 0.5% v/v Tween 20). Bound IgE Abs were detected with 1/15 diluted ^{125}I -labeled rabbit anti-human IgE Abs (RAST; Phadia), visualized by autoradiography, and quantified using a gamma counter (Wizzard Automatic Gamma Counter; Wallac).

For immunoblot inhibition experiments, 5 $\mu\text{g}/\text{cm}$ gel of purified natural parvalbumin (25) was separated by SDS-PAGE (24) and blotted onto nitrocellulose (Schleicher & Schuell; Ref. 26). Nitrocellulose strips were then exposed to the serum from a fish-allergic patient (patient 22), which had been preincubated with recombinant parvalbumin, with the parvalbumin mutants and, for control purposes, with an immunologically unrelated protein (BSA) (10 $\mu\text{g}/\text{ml}$ per 1/10 diluted serum). Bound IgE Abs were detected with the ^{125}I -labeled anti-human IgE Abs (Phadia).

Basophil histamine release assay and skin testing

Granulocytes were isolated from heparinized blood samples of a fish-allergic patient (patient 22) by dextran sedimentation (27). Cells were incubated with increasing concentrations of recombinant carp parvalbumin, the parvalbumin mutants, anti-human IgE Ab, or buffer as described previously (27). Liberated histamine was measured in the cell-free supernatants by radioimmunoassay (Immunotech). Results are expressed as mean values of

Table I. Clinical and serological characterization of patients

| Patient | Fish-Related Symptoms | Total IgE (kU/L) | Fish-Specific IgE Binding as Determined by CAP-FEIA ^a | |
|---------|---|------------------|--|-------|
| | | | Class | kUA/L |
| 1 | Urticaria, rhinitis, conjunctivitis | 784 | 2 | 3.2 |
| 2 | Rhinitis | 1,085 | 3 | 4.8 |
| 5 | Urticaria, rhinitis, conjunctivitis | 826 | 3 | 9.2 |
| 7 | Asthma, urticaria, rhinitis, conjunctivitis | 156 | 3 | 8.5 |
| 8 | Asthma, urticaria, rhinitis | 895 | 3 | 14.1 |
| 15 | Rhinitis | 183 | 3 | 11.1 |
| 22 | Asthma, urticaria, rhinitis | >2,000 | 3 | 8.0 |
| 25 | Asthma, urticaria, gastrointestinal reactions | 546 | 3 | 9.1 |

^a Fish-specific IgE were determined to cod extract (f3) using the CAP-FEIA system.

triplicate determinations and represent the percentage of total histamine, which was determined in cell lysates after freeze thawing of the cells (27).

Skin prick tests were performed in another fish-allergic patient (patient 25) with 20 μ l of aliquots of four concentrations (1 μ g/ml, 4 μ g/ml, 16 μ g/ml, and 32 μ g/ml) of recombinant carp parvalbumin or Mut-CD/EF, freshly diluted from a stock solution of 100 μ g/ml in normal saline, as well as of commercial prick solutions (cod extract, sardine extract, histamine, and sodium chloride; Stallergenes), according to standard procedures (28). The process of prick testing with recombinant allergens has been approved by the local ethics committee, and the patient gave written informed consent.

Immunization of mice and determination of Ag-specific IgG1 Ab levels by ELISA

Eight-week-old BALB/c mice (Charles River Breeding Laboratories) were maintained in the animal care unit of the Department of Pathophysiology (Medical University of Vienna) according to the local guidelines for animal welfare. The animals received s.c. injections in the neck of 10 μ g of aluminum hydroxide-adsorbed Mut-CD/EF. Mice were immunized three times in 4-wk intervals. Sera were obtained via bleeding from the tail vein and stored at -20°C until analysis (29).

Mouse IgG1 and IgE responses were measured by ELISA, as described previously (29): in brief, ELISA plates (Nunc Maxisorb) were coated with rCyp c 1 (4 μ g/ml in 0.1 M sodium bicarbonate (pH 9.6)) and blocked with 1% (w/v) human serum albumin in TBST. Plates were incubated with mouse sera diluted 1/1000 (IgG1) or 1/10 (IgE) in TBST for measurement of specific Ab levels. Bound IgG1 and IgE Abs were detected by incubating first with a monoclonal rat anti-mouse IgG1 or anti-mouse IgE Ab, respectively (BD Pharmingen; diluted 1/1000 in TBST), and then with HRP-coupled goat anti-rat antiserum (Amersham Biosciences) diluted 1/1000 in TBST. The color reaction was started by addition of 1.7 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich) in 60 mM citric acid, 77 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, and 3 mM H_2O_2 . OD levels were measured in an ELISA reader (Dynatech Laboratories) at 405 nm. All determinations were conducted in duplicates and results were expressed as mean values.

The biological activity of Cyp c 1-specific mouse IgE Abs was investigated in rat basophil leukemia (RBL)³ cell experiments (performed as described in Ref. 30), where the capability of Cyp c 1-specific IgE Abs to induce degranulation of RBL cells after contact with Cyp c 1 was analyzed.

Analysis of mouse anti-Mut-CD/EF Abs for cross-reactivity with parvalbumins from various fish species by immunoblotting

Protein extracts from carp, pikeperch, trout, and tuna were prepared, separated by SDS-PAGE, and blotted onto nitrocellulose as described previously (31). After blocking in PBST (PBS (pH 7.5) containing 0.5% v/v Tween 20), nitrocellulose strips were incubated with 1/1000 diluted mouse anti-Mut-CD/EF sera and, for control purposes, with the corresponding preimmune sera. Bound mouse Abs were detected with 1/1000 diluted rabbit anti-mouse Abs (Jackson ImmunoResearch Laboratories), followed by incubation with ¹²⁵I-labeled donkey anti-rabbit antiserum (Amersham Biosciences) and visualized by autoradiography.

ELISA competition assay for analyzing the inhibition of allergic patients' IgE binding to wild-type parvalbumin by Mut-CD/EF-induced IgG Abs

The ability of mouse Abs raised against purified Mut-CD/EF to inhibit the binding of patients' IgE to recombinant wild-type parvalbumin was examined in ELISA competition experiments. For this purpose, ELISA plate-bound wild-type parvalbumin (1 μ g/ml) was preincubated with anti-Mut-CD/EF antiserum or, for control purposes, with the corresponding preimmune serum (both 1/50 diluted in TBST). After incubation with 1/6 diluted sera from fish-allergic patients (patients 15, 22, 23, 24), bound human IgE was detected with HRP-coupled goat anti-human IgE Abs (1/2,500 diluted in TBST; Kirkegaard & Perry Laboratories). The color reaction was performed and quantified as described above for the experiment with the HRP-coupled anti-rat antiserum.

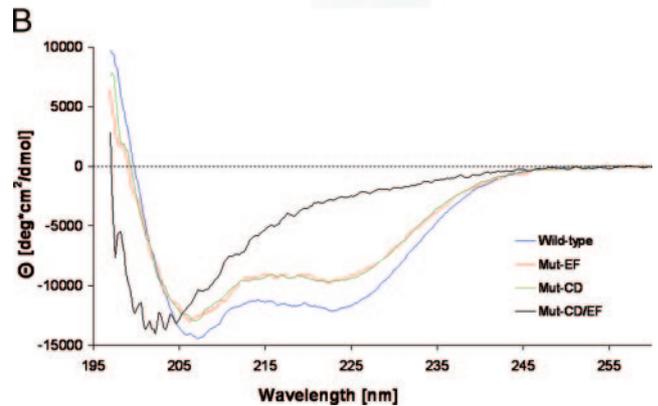
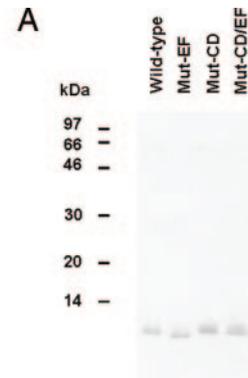


FIGURE 2. A, Coomassie brilliant blue-stained SDS-PAGE of purified recombinant parvalbumin (Wild-type) and of the parvalbumin mutants (Mut-EF, Mut-CD, Mut-CD/EF). B, Far-UV circular dichroism analysis of purified recombinant parvalbumin (Wild-type: blue) and the parvalbumin mutants (Mut-EF, red; Mut-CD, green; Mut-CD/EF, black). The mean residue ellipticity (Θ) (y-axis) is shown at given wavelengths (x-axis).

Results

Introduction of point mutations into both calcium-binding domains of parvalbumin leads to conformational changes

Based on our previous observations that calcium depletion reduced the IgE-binding capacity of parvalbumin (22, 31), we engineered hypoallergenic parvalbumin derivatives by site-directed mutagenesis of the two calcium-binding EF-hand domains of the molecule. To generate calcium-deficient parvalbumin mutants, we replaced the cation-coordinating aspartates at the first and third position of the calcium-binding loops by nonpolar alanine residues. These point mutations were introduced in either the first (Mut-CD), the second (Mut-EF), or both (Mut-CD/EF) of the calcium-binding domains of parvalbumin (Fig. 1).

Recombinant wild-type parvalbumin and the three mutants (Mut-CD, Mut-EF, Mut-CD/EF) were expressed in *E. coli* as soluble, nonfusion proteins and purified by ion exchange chromatography (wild-type parvalbumin, Mut-CD, Mut-EF). The double mutant (Mut-CD/EF) was further purified by hydrophobic interaction chromatography. Purity and migration behavior of the proteins was assessed by SDS-PAGE (Fig. 2A). Comparison of the proteins by circular dichroism analysis (Fig. 2B) revealed that the far-UV spectra of the proteins with mutations in one of the calcium-binding domains (Mut-CD and Mut-EF) showed similar characteristics as the wild-type protein. These spectra with minima at 208 nm and 227 nm and a maximum below 200 nm are typical for folded proteins with a considerable amount of α -helical secondary structure. The spectrum of the protein with mutations in both calcium-binding domains (Mut-CD/EF) differed significantly from the spectra of the other proteins. It exhibited a single minimum at \sim 202 nm, indicating an unstructured protein in random coil conformation.

³ Abbreviation used in this paper: RBL, rat basophil leukemia.

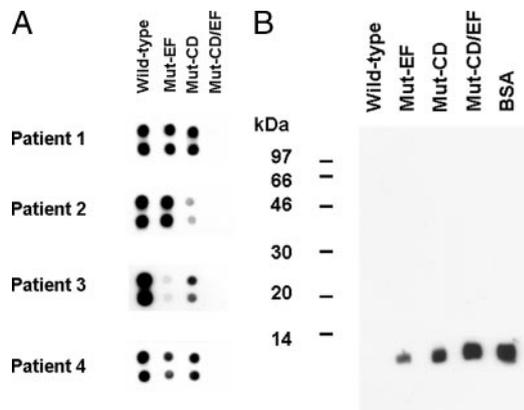


FIGURE 3. A, IgE reactivity of recombinant parvalbumin (Wild-type) and of the parvalbumin mutants (Mut-EF, Mut-CD, Mut-CD/EF). Nitrocellulose-dotted duplicates of the proteins were exposed to sera from four fish-allergic patients, and bound IgE Abs were detected with anti-human IgE Abs. B, Inhibition of IgE binding to nitrocellulose-blotted natural carp parvalbumin after preincubation of the serum from a fish-allergic patient (patient 22) with recombinant parvalbumin (Wild-type), with the parvalbumin mutants (Mut-EF, Mut-CD, Mut-CD/EF), or with the unrelated control protein BSA. Molecular weights are indicated in the left margin.

Parvalbumin mutants exhibit reduced IgE reactivity

The IgE-binding capacity of the three parvalbumin mutants was compared with the IgE reactivity of wild-type parvalbumin in two types of assays. First, we studied IgE reactivity to nondenatured, immobilized proteins in dot blot assays (Fig. 3A). Sera from fish-allergic patients showed strong IgE reactivity to the wild-type allergen, a varying reduction of IgE reactivity toward Mut-CD and Mut-EF, and no IgE binding to Mut-CD/EF (Fig. 3A).

For a more extensive and quantitative analysis of the IgE-binding capacity of the parvalbumin derivatives, dot blot assays were performed with sera from 21 fish-allergic patients and IgE binding was quantified by gamma counting (Table II). The quantitative data demonstrated that modifications in one of the calcium-binding domains (Mut-CD or Mut-EF) resulted in a varying pattern of IgE recognition. The majority of patients, 20 in case of Mut-CD and 14 in case of Mut-EF, showed reduced IgE binding to the two mutants (Mut-EF, 3.1–93.5% reduction of the IgE reactivity; Mut-CD, 7.4–84.6% reduction). As described previously for other calcium-binding allergens, we also noted that certain patients displayed increased IgE reactivities to Mut-CD or Mut-EF as compared with wild-type carp parvalbumin. This increase in IgE-binding capacity was most pronounced for patient 8, whose IgE reactivity to Mut-EF was 3-fold more intense than that to the wild-type allergen.

In contrast to the single mutants, the double mutant (Mut-CD/EF) showed in each of the tested patients a strongly reduced IgE-binding capacity, which on average was <5% of the IgE reactivity of wild-type parvalbumin.

Results from the binding studies to immobilized proteins were confirmed by a fluid phase inhibition test (Fig. 3B). In this assay, the serum from a fish-allergic patient (patient 22) was preadsorbed with the wild-type or mutant proteins and then exposed to nitrocellulose-blotted natural carp parvalbumin. As shown in Fig. 3B, IgE binding to the natural allergen was completely inhibited by addition of recombinant wild-type protein. Mut-CD and Mut-EF inhibited IgE reactivity much less than the wild-type protein, and preincubation with Mut-CD/EF had no effect on the IgE binding to natural parvalbumin, confirming the strong reduction of IgE epitopes on Mut-CD/EF.

Table II. IgE reactivity of fish-allergic patients to dot-blotted recombinant parvalbumin and to the parvalbumin mutants^a

| Patient | Wild-Type cpm | Mut-EF | | Mut-CD | | Mut-CD/EF | |
|---------|------------------|--------|-----------------------------|--------|-----------------------------|-----------|-----------------------------|
| | | cpm | Percent Wild Type (%) | cpm | Percent Wild Type (%) | cpm | Percent Wild Type (%) |
| 1 | 738.2 | 464.0 | 62.9 | 496.0 | 67.2 | 46.6 | 6.3 |
| 2 | 751.8 | 882.1 | 117.3 | 123.5 | 16.4 | 10.8 | 1.4 |
| 3 | 1442.8 | 45.2 | 3.1 | 165.2 | 11.4 | 17.1 | 1.2 |
| 4 | 473.4 | 169.6 | 35.8 | 208.1 | 44.0 | 19.0 | 4.0 |
| 5 | 65.9 | 61.6 | 93.5 | 8.6 | 13.1 | 2.6 | 3.9 |
| 6 | 16.2 | 33.3 | 205.6 | 13.7 | 84.6 | 8.6 | 53.1 |
| 7 | 82.5 | 85.5 | 103.6 | 26.0 | 31.5 | 0.6 | 0.7 |
| 8 | 28.9 | 106.0 | 366.8 | 21.9 | 75.8 | 8.6 | 29.8 |
| 9 | 62.0 | 31.3 | 50.5 | 47.5 | 76.6 | 9.6 | 15.5 |
| 10 | 92.2 | 23.2 | 25.2 | 26.0 | 28.2 | 24.5 | 26.6 |
| 11 | 33.7 | 29.3 | 86.9 | 14.8 | 43.9 | 4.6 | 13.6 |
| 12 | 72.7 | 60.6 | 83.4 | 87.5 | 120.4 | 4.6 | 6.3 |
| 13 | 329.2 | 193.9 | 58.9 | 128.4 | 39.0 | 1.6 | 0.5 |
| 14 | 194.6 | 104.0 | 53.4 | 48.5 | 24.9 | 12.6 | 6.5 |
| 15 | 438.4 | 374.6 | 85.4 | 113.1 | 25.8 | 1.4 | 0.3 |
| 16 | 75.7 | 138.3 | 182.7 | 21.9 | 28.9 | 1.6 | 2.1 |
| 17 | 258.0 | 206.0 | 79.8 | 110.0 | 42.6 | 4.6 | 1.8 |
| 18 | 172.2 | 62.6 | 36.3 | 12.7 | 7.4 | 12.6 | 7.3 |
| 19 | 192.7 | 344.3 | 178.7 | 60.8 | 31.6 | 5.6 | 2.9 |
| 20 | 211.2 | 75.7 | 35.8 | 131.5 | 62.3 | 6.6 | 3.1 |
| 21 | 84.8 | 89.4 | 105.4 | 70.1 | 82.7 | 41.5 | 48.9 |
| Mean | 277.0 | 170.5 | 61.6 | 92.2 | 33.3 | 11.7 | 4.2 |

^a IgE reactivity was quantified by gamma counting. Results are displayed in counts per minute (cpm) and as percentage of IgE binding of wild-type parvalbumin (percent wild type).

Parvalbumin mutants show reduced allergenic activity in vitro and in vivo

To analyze the allergenic activity of the parvalbumin mutants, peripheral blood basophils of a fish-allergic patient (patient 22) were incubated with different concentrations of the recombinant wild-type and mutant proteins (Fig. 4). Recombinant wild-type Cyp c 1 induced a dose-dependent release of histamine from the basophils. Mut-CD or Mut-EF induced ~10-fold less histamine release than the wild-type protein (Fig. 4). Mut-CD/EF did not induce any release of histamine up to a concentration of 1 μg/ml.

The reduced allergenic activity of Mut-CD/EF was confirmed in an in vivo skin prick test experiment in another fish-allergic patient

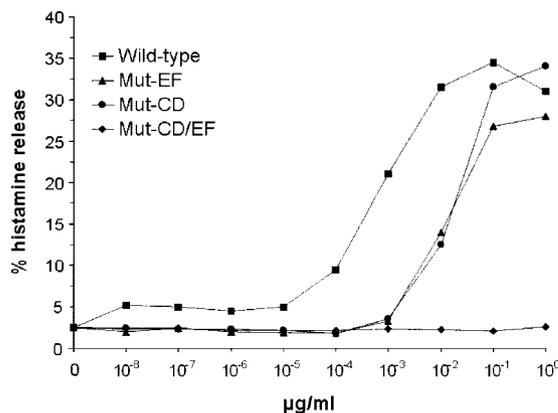


FIGURE 4. Allergenic activity of purified recombinant parvalbumin (Wild-type) and of the parvalbumin mutants (Mut-EF, Mut-CD, Mut-CD/EF). Induction of histamine release from basophils of a fish-allergic patient (patient 22) by incubation with various concentrations (x-axis) of the recombinant proteins. The percentage of total histamine released into the supernatant is displayed on the y-axis.

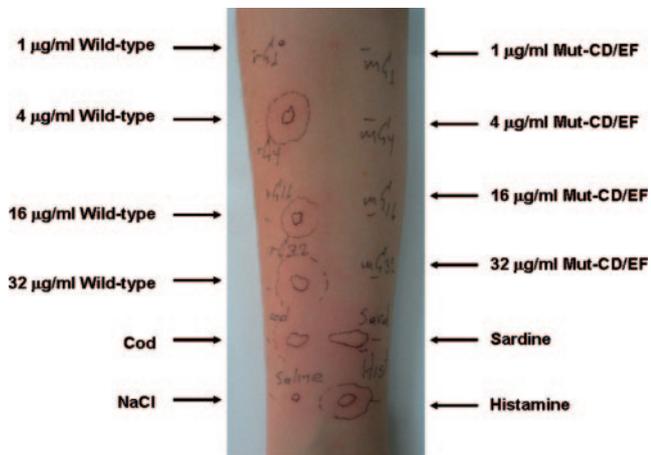


FIGURE 5. Induction of immediate-type skin reactions with recombinant parvalbumin (Wild-type) and mutant Mut-CD/EF. A fish-allergic patient (patient 25) was skin prick tested with different concentrations (1 $\mu\text{g/ml}$, 4 $\mu\text{g/ml}$, 16 $\mu\text{g/ml}$, 32 $\mu\text{g/ml}$) of the recombinant proteins, with cod extract (Cod), sardine extract (Sardine), histamine hydrochloride (Histamine), and sodium chloride (NaCl). The wheal reactions are marked with a ballpoint pen.

(patient 25; Fig. 5). After application of different concentrations of recombinant wild-type parvalbumin and of the double mutant, Mut-CD/EF, on the forearm of the patient, the individual showed pronounced immediate-type skin reactions to rCyp c 1 wild-type protein already at low allergen concentrations (1 $\mu\text{g/ml}$ or 4 $\mu\text{g/ml}$; Fig. 5). In contrast, Mut-CD/EF induced no skin reactions even when applied at the highest concentration (32 $\mu\text{g/ml}$). Cod and sardine extracts and histamine, which were applied for control purposes, also induced pronounced immediate-type skin reactions in the patient. Comparable skin prick test results were obtained in eight additional fish-allergic patients (28).

Immunization with Mut-CD/EF induces IgG Abs that cross-react with wild-type carp parvalbumin and with parvalbumins from various fish species

To analyze whether immunization with Mut-CD/EF induces IgG Abs that cross-react with parvalbumins from important fish species, BALB/c mice were immunized with purified Mut-CD/EF. ELISA analyses revealed that in each of the immunized mice Mut-CD/EF induced specific IgG₁ Abs, which recognized Mut-CD/EF and also the recombinant wild-type allergen (data not shown). Immunoblot analyses showed that Mut-CD/EF-specific mouse IgG Abs cross-reacted with natural Cyp c 1 and with parvalbumins

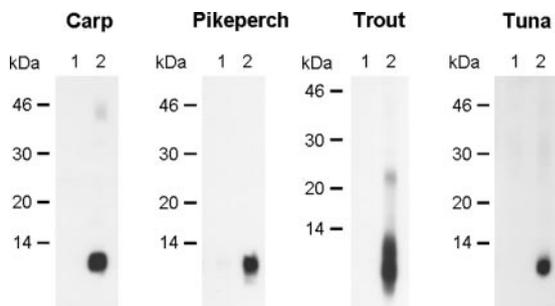


FIGURE 6. Reactivity of mouse anti-Mut-CD/EF IgG Abs with natural carp parvalbumin and with parvalbumins from other fish species. Pre-immune serum (lanes 1) and immune serum (lanes 2) were tested for IgG reactivity to nitrocellulose-blotted protein extracts from carp, pikeperch, trout, and tuna. Molecular weights are indicated in the left margins.

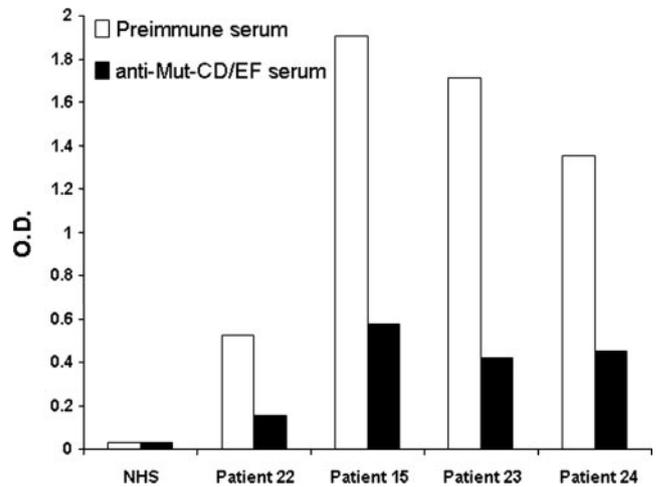


FIGURE 7. Mouse anti-Mut-CD/EF Abs inhibit patients' IgE binding to recombinant parvalbumin (Wild-type). ELISA plate-bound recombinant parvalbumin was preincubated with 1/50 diluted mouse anti-Mut-CD/EF antiserum or with the corresponding preimmune serum and subsequently exposed to sera from four fish-allergic patients or from a nonatopic individual (NHS). Mean OD values corresponding to IgE binding were measured by ELISA.

present in fresh and salt water fish (carp, pikeperch, trout, and tuna) (Fig. 6). Mut-CD/EF-immunized mice developed a weak IgE response to the wild-type allergen, but loading of RBL cells with these Abs and subsequent challenge with the allergen failed to induce any relevant degranulation (data not shown).

IgG Abs induced by immunization with Mut-CD/EF inhibit patients' IgE binding to the wild-type allergen

To further investigate whether mouse anti-Mut-CD/EF Abs are able to block allergic patients' IgE binding to recombinant wild-type parvalbumin, ELISA competition experiments were performed. As exemplified in Fig. 7, IgE binding of four fish-allergic patients (patients 15, 22, 23, 24) to wild-type parvalbumin was reduced after preincubation of ELISA plate-bound wild-type parvalbumin with anti-Mut-CD/EF antiserum (■), but not after preincubation with the mouse preimmune serum (□). Inhibition levels obtained with Mut-CD/EF-specific mouse serum ranged from 67 to 76%.

Discussion

In this study, we constructed three hypoallergenic variants of the major fish allergen parvalbumin for immunotherapy. The cDNA coding for carp parvalbumin, Cyp c 1, a protein which contains the majority of IgE epitopes present in fresh water and salt water fish, was used as a template for site-directed mutagenesis of the calcium-binding domains of parvalbumin. This strategy was chosen because we previously observed that depletion of calcium from parvalbumin reduces its IgE reactivity (22). The circular dichroism spectra of the recombinant parvalbumin derivatives showed that the overall structure of the two derivatives containing mutations in only one calcium-binding domain (single mutants; Mut-CD and Mut-EF) still resembled the mainly α -helical structure of wild-type parvalbumin, whereas the derivative containing mutations in both calcium-binding domains (double mutant; Mut-CD/EF) displayed a significantly decreased α -helical content and a transition into a random coil, unfolded conformation. The loss of fold was associated with a reduction of IgE Ab reactivity. The two predominantly α -helical single mutants exhibited only a modest reduction of IgE reactivity. However, the unfolded double mutant, Mut-CD/EF,

showed a consistent reduction of IgE reactivity of ~95% and a strongly reduced allergenic activity. IgE epitopes of Cyp c 1, a prominent class I food allergen, capable of inducing severe systemic anaphylactic reactions, thus seem to be of the conformational type. In case of other class I food allergens, which frequently elicit life-threatening anaphylactic reactions, such as the major peanut allergens Ara h 1, Ara h 2, and Ara h 3 (32–34), the major shrimp allergen, Pen a 1 (35), and major milk allergens (36, 37), it has been reported that IgE-binding primarily occurs to linear epitopes (reviewed in Ref. 12). Cyp c 1 seems to represent an exception to the rule and also illustrates that conformational epitopes may play a role in anaphylactic reactions to food allergens. The latter may be due to the fact that the conformation of Cyp c 1 is highly resistant to thermal denaturation and that the protein even refolds after cooking (25). This remarkable behavior, which has also been described for certain highly stable calcium-binding pollen allergens (38), may be attributed to stabilization of the protein structure by calcium binding.

Even though the Mut-CD/EF derivative represents an essentially unfolded molecule, which basically lacks the IgE-binding epitopes of wild-type parvalbumin, it is still immunogenic. Immunization of mice with Mut-CD/EF induced IgG Abs, which recognized the unfolded immunogen as well as the wild-type allergen and inhibited the binding of allergic patients' IgE to wild-type parvalbumin. Due to the fact that Mut-CD/EF-induced IgG Abs apparently recognized sequential/denatured epitopes on the unfolded immunogen Mut-CD/EF, we think that these Abs inhibit IgE binding to the wild-type allergen mainly through steric hindrance. We suggest that Mut-CD/EF may be used for immunotherapy similarly as hypoallergenic derivatives of the major birch pollen allergen Bet v 1, which have already been successfully used for immunotherapy of birch pollen allergy in birch pollen-allergic patients. The recombinant Bet v 1 derivatives with preserved T cell epitopes showed reduced allergenic activity and induced in patients protective IgG Abs, which inhibited immediate-type allergic reactions and reduced boosts of IgE production upon allergen contact (39).

Because we have exchanged only four amino acids in the Mut-CD/EF derivative, we expect that the T cell epitopes of the parvalbumin wild-type allergen are essentially preserved in the mutant. Mut-CD/EF may therefore also be used for T cell-based immunotherapy forms such as the induction of tolerance (reviewed in Refs. 40 and 41). Due to its strongly reduced allergenic activity, the protein exhibits a reduced risk of inducing anaphylactic reactions and thus may also be used for mucosal application (reviewed in Ref. 42). Furthermore, it will also be possible to administer the protein together with new adjuvants (reviewed in Ref. 43) or conjugated to immunostimulatory sequences (Ref. 44 and reviewed in Ref. 45). Also, DNA vaccination with DNA encoding the hypoallergenic Cyp c 1 mutant may be considered as an approach for treatment (46). We therefore think that the Mut CD/EF parvalbumin variant will represent the basis for new forms of vaccination against fish allergy.

Acknowledgment

We thank Nadja Balic for excellent technical assistance.

Disclosures

R. Valenta is an allergy consultant for Phadia and Biomay and has also received research grants from both companies.

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