

Recombinant Carp Parvalbumin, the Major Cross-Reactive Fish Allergen: A Tool for Diagnosis and Therapy of Fish Allergy¹

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IgE-mediated reactions to fish allergens represent one of the most frequent causes of food allergy. We have constructed an expression cDNA library from carp (*Cyprinus carpio*) muscle in phage λ gt11 and used serum IgE from a fish allergic patient to isolate 33 cDNA clones that coded for two parvalbumin isoforms (Cyp c 1.01 and Cyp c 1.02) with comparable IgE binding capacities. Both isoforms represented calcium-binding proteins that belonged to the β -lineage of parvalbumins. The Cyp c 1.01 cDNA was overexpressed in *Escherichia coli*, and rCyp c 1.01 was purified to homogeneity. Circular dichroism analysis and mass spectroscopy showed that rCyp c 1.01 represented a folded protein with mainly α -helical secondary structure and a molecular mass of 11,416 Da, respectively. rCyp c 1.01 reacted with IgE from all fish-allergic patients tested ($n = 60$), induced specific and dose-dependent basophil histamine release, and contained most of the IgE epitopes (70%) present in natural allergen extracts from cod, tuna, and salmon. Therefore, it may be used to identify patients suffering from IgE-mediated fish allergy. The therapeutic potential of rCyp c 1.01 is indicated by our findings that rabbit Abs raised against rCyp c 1.01 inhibited the binding of IgE ($n = 25$) in fish-allergic patients to rCyp c 1.01 between 35 and 97% (84% mean inhibition) and that depletion of calcium strongly reduced IgE recognition of rCyp c 1.01. The latter results suggest that it will be possible to develop strategies for immunotherapy for fish allergy that are based on calcium-free hypoallergenic rCyp c 1.01 derivatives. *The Journal of Immunology*, 2002, 168: 4576–4584.

Together with milk, egg, peanuts, tree nuts, and shellfish, fish is among the most important allergen sources causing IgE-mediated food hypersensitivity (1–3). Although not a major health problem on a world-wide basis, fish allergy can reach a prevalence of 1 per 1000 individuals in fish-eating and fish-processing countries (4). Ingestion of fish, inhalation of vapors generated during cooking, and skin contact can cause a variety of IgE-mediated clinical symptoms in sensitized patients. These symptoms comprise acute urticaria, angioedema, atopic dermatitis, respiratory (rhinoconjunctivitis, asthma) and gastrointestinal (diarrhea, vomiting) symptoms, and, in some cases, fatal anaphylaxis (4–6).

Not only is fish allergy a typical immunologically mediated hypersensitivity disease, but it also played an important role in the elucidation of pathomechanisms underlying IgE-mediated allergies. In 1921, Prausnitz and Küstner (50) performed a classical experiment by transferring serum from a fish allergic patient into the skin of a nonatopic individual and showed that subsequent

exposure of the nonatopic subject's skin to fish led to an allergic reaction. This classical experiment demonstrated that immediate-type hypersensitivity requires three components: allergens, allergen-specific factors that are present only in the serum of atopic patients, and tissue components that can be found in every individual. More than forty years later, the allergen-specific serum factors could be identified as a novel class of Igs, termed IgE, which bind via specific receptors to effector cells (e.g., mast cells and basophils) as well as to APC (B cells, monocytes, and dendritic cells). Almost at the same time research groups started to work on the molecular characterization of allergens (reviewed in Ref. 7).

Parvalbumins from fish represent extremely abundant and stable allergens and therefore were among the first identified allergen molecules (8–10). Parvalbumins are small (12-kDa) calcium-binding proteins with a remarkable resistance to heat, denaturing chemicals, and proteolytic enzymes (11). They are characterized by the presence of three typical helix-loop-helix Ca^{2+} binding domains, termed EF-hands (12–14). Two of these EF-hand motifs are capable of binding Ca^{2+} as well as Mg^{2+} , while the first, silent domain forms a cap that covers the hydrophobic surface of the pair of functional domains (15, 16). Parvalbumins are present in high amounts in the white muscles of lower vertebrates (17) and in lower amounts in fast twitch muscles of higher vertebrates (18), where they function in calcium buffering and may be involved in the relaxation process of muscles (19). Based on amino acid sequence data the parvalbumin protein family can be subdivided into two evolutionary distinct lineages: the α group, consisting of less acidic parvalbumins with isoelectric points at or above pI 5.0, and the β group, consisting of more acidic parvalbumins with isoelectric points at or below pI 4.5 (20).

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Resistance to boiling and to enzymes of the gastrointestinal tract may, in fact, be a predisposing factor that these proteins can act as potent sensitizing agents for >95% of fish allergic patients (6, 21–24). It was further shown that patients who mount IgE Abs against one parvalbumin will cross-react with the homologous proteins from other fish species (24), which demonstrates the importance of parvalbumins as cross-reactive fish allergens and explains why allergic individuals exhibit clinical symptoms upon contact with various fish species. IgE competition experiments performed with purified carp parvalbumin indicated that this molecule contained a large portion of IgE epitopes present in various fish species (25).

To obtain IgE-reactive recombinant carp parvalbumin that can be used for diagnosis and perhaps treatment of fish allergy we constructed an expression cDNA library from carp muscle and searched with IgE Abs of fish allergic patients for cDNA clones coding for IgE-reactive parvalbumin forms. The production and characterization of the first IgE-reactive recombinant fish parvalbumin mimicking the properties of the corresponding natural allergen are reported in this study.

Materials and Methods

Human sera and Abs

Sera were obtained from patients with a positive case history of type I allergy to fish, who experienced at least one of the typical clinical symptoms (dermatitis, urticaria, angioedema, diarrhea, asthma, or anaphylactic reaction) after contact with fish proteins. For verification of diagnosis, fish-specific IgE Abs were determined using the CAP-FEIA System (Pharmacia, Uppsala, Sweden). IgE competition experiments comparing rCyp c 1.01 and natural fish extracts were performed with sera from patients who cross-reacted with several fish species. A mAb against carp parvalbumin was purchased from Sigma-Aldrich (clone PA-235; St. Louis, MO).

Construction of a carp muscle cDNA library and isolation of IgE-reactive cDNAs and sequence analysis

Total RNA was isolated from carp muscle tissue according to the guanidium isothiocyanate method described by Davis et al. (26). Poly(A)⁺ mRNA, enriched by chromatography on oligo(dT)-cellulose, was used for cDNA synthesis, which was conducted with oligo(dT) primers using a cDNA synthesis kit (Amersham, Little Chalfont, U.K.) following the manufacturer's instructions. The double-stranded cDNA was methylated, ligated to *Eco*RI linkers, digested with *Eco*RI, and inserted into dephosphorylated λ gt11 *Eco*RI-cut arms. Packaging was performed using the Amersham in vitro packaging module. The expression library was screened with serum IgE from a fish-allergic patient who had experienced systemic anaphylactic reactions after ingestion of fish. A total of 33 IgE-reactive clones were isolated, subcloned into plasmid pUC18, and sequenced by the dideoxynucleotide chain termination method (27) using a T7 sequencing kit (Pharmacia). Analysis of the sequences and comparison with the sequences deposited in GenBank, European Molecular Biology Laboratory, DNA Data Base in Japan, and Protein Data Bank libraries showed that all the clones coded for parvalbumins and revealed the presence of two carp parvalbumin isogenes. A multiple sequence alignment of the deduced amino acid sequences with parvalbumin proteins retrieved from the SwissProt database was produced with ClustalW (28). Protein secondary structure predictions based on position-specific scoring matrices were performed as described by Jones (29).

Three-dimensional structural modeling

The rCyp c 1.01 structure was generated by homology modeling (30, 31) using the crystal structures of a carp parvalbumin with an isoelectric point (pI) of 4.25 (data base entry code P02618) (32) and silver hake parvalbumin (pI of 4.2; data base entry code P56503) (33) as templates. The energy-minimized model was prepared with Swissmodel (30, 31) and drawn using the programs Molscript (34) and Raster3D (35).

Expression and purification of recombinant carp parvalbumin

The IgE binding capacity of the phage clones expressing full-length parvalbumin and parvalbumin fragments was investigated using a plaque lift assay (36). Because both parvalbumin isoforms exhibited comparable IgE reactivity with sera from several fish allergic patients, the DNA coding for

Cyp c 1.01 was PCR amplified and subcloned into the *Nde*I/*Eco*RI site of expression vector pET-17b (Novagen, Madison, WI). To avoid internal cutting of the cDNA, an internal *Eco*RI site at the 5' end of the parvalbumin clone had to be mutated. This was achieved using the following oligonucleotide primers for PCR amplification: a primer specific for the 5' end of the clone: 5'-GG GCA TTC CAT ATG GCA TTC GCT GGT ATT CTG AAT GAT GCT G-3', in which the *Eco*RI site was changed (underlined) and which contained an *Nde*I site (italics) and a primer complementary to the 3' end with an *Eco*RI site (italics): 5'-GG GAA TTC TTA TGC CTT GAC CAG GGC-3'. Recombinant parvalbumin was expressed in liquid cultures of *Escherichia coli* BL21(DE3) after induction of protein synthesis with isopropyl β -D-thiogalactoside (0.5 mM). The majority of the protein was found in the soluble fractions of the bacterial extracts. Therefore, *E. coli* cells were resuspended in PBS (pH 7.5) containing 1 mM PMSF and were mechanically disrupted by sonication. After the insoluble material had been removed by centrifugation at 20,000 $\times g$ for 30 min, recombinant parvalbumin was further enriched in the supernatant by ammonium sulfate precipitation (60%, w/v) of contaminating proteins. Ammonium sulfate was removed by dialysis against distilled water, and the proteins present in the supernatant were lyophilized, dissolved in 10 mM Tris (pH 7.5), and applied to a DEAE-cellulose-Sepharose column (DEAE Sepharose Fast Flow column; Pharmacia). Fractions containing purified parvalbumin were eluted with a linear salt gradient (0–0.5 M NaCl in 10 mM Tris (pH 7.5)) and dialyzed against distilled water.

Matrix-assisted laser desorption and ionization-time of flight and circular dichroism (CD)⁴ analysis of purified recombinant parvalbumin

Laser desorption mass spectra were acquired in a linear mode with a TOF Compact MALDI II instrument (Kratos, Manchester, U.K.; piCHEM, Research and Development, Graz, Austria). Samples were dissolved in 10% acetonitrile (0.1% trifluoroacetic acid), and α -cyano-4 hydroxycinnamic acid (dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid) was used as a matrix. For sample preparation a 1/1 mixture of protein and matrix solution was deposited onto the target and air-dried.

CD measurements were performed on a Jasco (Tokyo, Japan) J-715 spectropolarimeter with protein concentrations between 12.3–24.0 μ M using a 1-mm path-length quartz cuvette (Hellma, Mullheim, Baden, Germany) equilibrated at 20°C. Spectra were recorded with 0.2-nm resolution at a scan speed of 50 nm/min, and results were the average of three scans. The final spectra were corrected by subtracting the corresponding baseline spectrum obtained under identical conditions. Results are expressed as the mean residue ellipticity (θ) at a given wavelength.

Immunoblot analyses and calcium depletion experiments

Reactivities of recombinant carp parvalbumin to serum IgE from fish allergic patients and to an anti-parvalbumin mAb were determined by immunoblot analyses as described previously (25). For immunoblot inhibition experiments, sera from fish-allergic patients were preincubated with purified recombinant parvalbumin (10 μ g/ml of 1/10 diluted serum). Thereafter, nitrocellulose-blotted purified natural parvalbumin was incubated with the preabsorbed serum samples, and bound IgE was detected using ¹²⁵I-labeled anti-human IgE Abs (Pharmacia).

To investigate the effects of depletion of protein-bound Ca²⁺ on the IgE-binding capacity of rCyp c 1.01, nitrocellulose strips containing equal amounts of blotted recombinant protein were exposed to patients' sera in the presence of either 0.5 mM CaCl₂ or 5 mM EGTA. Bound Abs were detected with ¹²⁵I-labeled anti-human IgE Abs (Pharmacia). Reduction of IgE binding to parvalbumin was also quantified by gamma counting (Wizdard, Automatic Gamma Counter; Wallac, Uppsala, Sweden) of the nitrocellulose strips and was calculated as the percent inhibition = ((cpm_{Ca2+} – cpm_{EGTA})/cpm_{Ca2+}) $\times$ 100, where cpm_{Ca2+} and cpm_{EGTA} indicate IgE binding to the calcium-bound and calcium-free forms, respectively.

Quantitative IgE absorption assays

Sera from fish-allergic patients were preincubated with 5 μ g recombinant carp parvalbumin or, for control purposes, with 5 μ g BSA. Remaining serum IgE reactivity to cod, tuna, and salmon total fish extracts was measured using the CAP-FEIA System (Pharmacia). The percent inhibition of IgE binding to fish extracts after preabsorption with recombinant carp parvalbumin was calculated as ((cpm_{BSA} – cpm_{parv})/cpm_{BSA}) $\times$ 100, where cpm_{BSA} and cpm_{parv} indicate IgE binding after preabsorption with BSA and recombinant carp parvalbumin, respectively.

⁴ Abbreviation used in this paper: CD, circular dichroism.

ELISA for quantification of IgE and IgG subclass reactivities; ELISA competition assay for analyzing the inhibition of human IgE binding to rCyp c 1.01 by rCyp c 1.01-specific IgG

The prevalence of IgE and IgG subclass reactivity to recombinant carp parvalbumin or, for control purposes, to rPhl p 5, an immunologically unrelated timothy grass pollen allergen (37), was determined in sera from fish-allergic patients, grass pollen-allergic patients, and nonatopic individuals by ELISA. ELISA plates (Nunc Maxisorb, Roskilde, Denmark) were coated with the recombinant proteins (5 µg/ml in 0.1 M sodium bicarbonate (pH 9.6)) and blocked with 1% human serum albumin in TBST. Plates were incubated with sera diluted 1/5 in TBST for measurement of specific IgE and 1/20 for measurement of IgG1, IgG2, IgG3, and IgG4. Bound IgE Abs were detected by adding an alkaline phosphatase-coupled mouse anti-human IgE mAb (BD PharMingen, San Diego, CA) diluted 1/1000 in TBST, and the color reaction was developed by incubation with alkaline phosphatase substrate (Sigma-Aldrich). Bound IgG subclass Abs were detected by incubating first with monoclonal mouse anti-human IgG subclass-specific Abs (BD PharMingen) diluted 1/1000 in TBST and then with a HRP-coupled sheep anti-mouse antiserum (Amersham) diluted 1/2000 in TBST. The color reaction was started by addition of 1.7 mM 2,2'-azino-di-[3-ethyl-benzthiazolin-sulfonate] (Sigma-Aldrich) in 60 mM citric acid, 77 mM Na₂HPO₄·2H₂O, and 3 mM H₂O₂. ODs were measured in an ELISA reader (Dynatech, Denkendorf, Germany) at 405 nm. All determinations were conducted as duplicates, and results are expressed as mean values.

The ability of rabbit Abs raised against purified rCyp c 1.01 (Charles River Breeding Laboratories, Kisslegg, Germany) to inhibit the binding of patients' IgE to recombinant parvalbumin was examined by ELISA competition experiments as previously described (36). ELISA plate-bound rCyp c 1.01 (1 µg/ml) was preincubated with different concentrations of the anti-rCyp c 1.01 antiserum and, for control purposes, with dilutions of the corresponding preimmune serum. After incubation with 1/5 diluted sera from fish-allergic patients, bound IgE was detected with HRP-coupled goat anti-human IgE Ab (1/2500 diluted; Kirkegaard & Perry, Gaithersburg, MD). The color reaction was performed and quantified as described above for the experiments with the HRP-coupled sheep anti-mouse antiserum. The percent inhibition of IgE binding achieved by preincubation with the anti-rCyp c 1.01 antiserum was calculated as follows: % inhibition of IgE binding = 100 - (OD_s/OD_p) × 100, where OD_s and OD_p represent the extinction coefficients after preincubation with the rabbit serum and the preimmune serum, respectively.

Basophil histamine release assay

Granulocytes were isolated from heparinized blood samples of a fish-allergic patient by dextran sedimentation. Cells were incubated with increasing concentrations of recombinant carp parvalbumin, anti-human IgE Ab, or buffer as previously described (38). Liberated histamine was measured in the cell-free supernatants by RIA (Immunotech, Marseille, France).

Results

Isolation and characterization of cDNAs coding for the major carp allergen, a β-type parvalbumin

Approximately 380,000 plaques of the carp muscle cDNA expression library were screened with serum IgE from a fish-allergic patient. Sequencing of 33 independently obtained IgE-reactive cDNA clones revealed that they all coded for parvalbumin and demonstrated the presence of two distinct, highly homologous carp parvalbumin isoforms, designated Cyp c 1.01 and Cyp c 1.02 (Fig. 1; accession no. AJ292211 and AJ292212 in the EMBL Nucleotide Sequence Database). The open reading frames of both variants encode mature proteins of a size typical for parvalbumins of the β lineage, with a calculated molecular mass of 11.5 kDa and isoelectric points of 4.41 (Cyp c 1.01) and 4.77 (Cyp c 1.02). Computer-aided secondary structure analysis predicts six α-helices organized in three helix-loop-helix motifs (Fig. 2A). Such motifs are characteristic for the Ca²⁺ binding domains of the EF-hand family of Ca²⁺-binding proteins (39–41). A further search for sequence motifs revealed the presence of a protein kinase C phosphorylation site (aa 37–39) and three casein kinase II phosphorylation sites (aa 40–43, 79–82, and 92–95) in both isoforms.

Cyp c 1.01

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cgtttcacccctgcgaatccaaacccaacccctaagacaaccagaggctaatacATGGCA 60
                                     M A 2
TTCGCTGGAATCTGAATGATGCTGACACTCACTGCAGCCCTGCAGGGCTGCCAAGCTGCT 120
F A G I L N D A D I T A A L Q G C Q A A 22
GACTCCTCTGACTACAAGAGCTTCTTCGCGAAGGTGGTGTCTGTCTGCCAAGACTCCTGAT 180
D S F D Y K S F F A K V G L S A K T P D 42
GACATCAAGAAGGCCCTTGTCTGTCATTGACCAAGACAAGAGCGGCTTCATTGAGGAGGAT 240
D I K K A F A V I D Q D K S G F I E E D 62
GAGCTGAAACTGTTCTCTGCAGAACTTCTCTGCTGGCGCCAGGGCACTCACTGATGCAGAG 300
E L K L F L Q N F S A G A R A L T D A E 82
ACAAAGGCCCTTCTGAAAGCTGGAGACTCTGATGGTGTATGGCAAGATTGGAGTTGATGAG 360
T K A F L K A G D S D G D G K I G V D E 102
TTTGTGCGCCCTGGTCAAGGCATAAatggcacctgaccaacaacttttgctgacaatcagt 420
F A A L V K A * 109
atgtaacctgagatctccactttttatcggggtggctgattttttatcacacaattctaa 480
ctgtccactccatgctgcaccgggtccacaaatgactgtcaacgtccacacgggtctttccac 540
gttgtgatgatcatgtgtgtttctgtataaatctttgatgctgttttgggaaggacaa 600
tgagaaaataaacactatttttcattaaaaaataaataaataaataaataaataaataa 648
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Cyp c 1.02

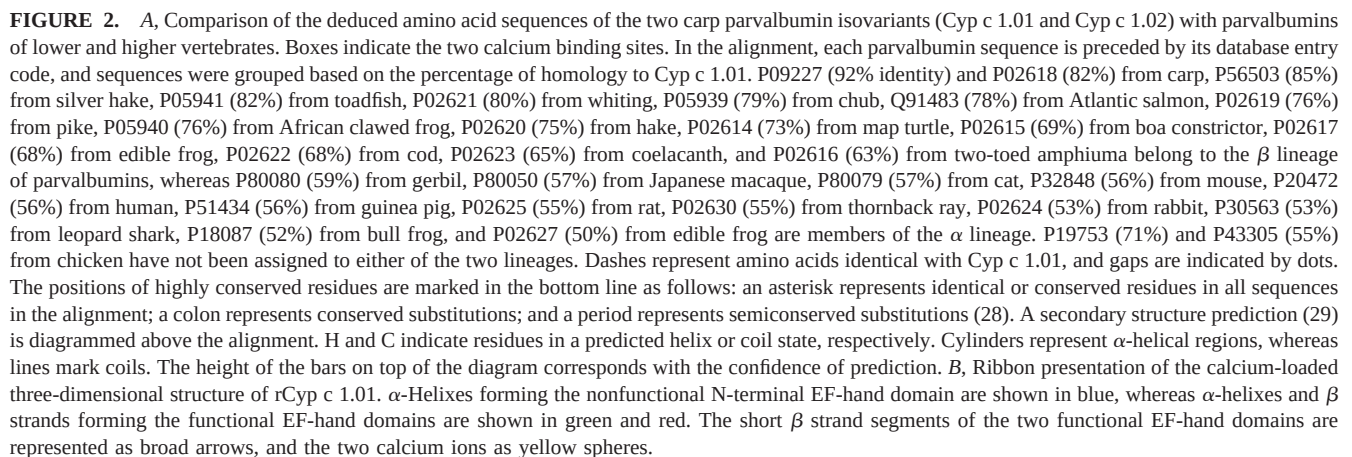
```
cgaaactaccttcagcaccacaaagacaaaagagcetaaagATGGCTTCGCGGAGTCTCTG 60
                                     M A F A G V L 7
AACGCGCTGACACTCACTGCAGCACTGGAGGCGATGCAAGGCTGCTGACTCCTTCAACCAC 120
N D A D I T A A L E A C K A A D S F N H 27
AAGACTTTCTTCGCGAAGGTGGTCTGACCAAGCTGCTGATGATGTGAAGAAGGCT 180
K T F F A K V G L T S K S A D D V K K A 47
TTCGCAATCTGATGACAGGACAAGAGTGGCTTCATTGAGGAGGATGAACCTGAACTGTTTC 240
F A I I D Q D K S G F I E E D E L K L F 67
CTGCAGAAGCTTCAAGGCTGGGGCCAGGGCACTGACTGACGGCGAGACCAAGACTTCTCTG 300
L Q N F K A G A R A L T D G E T K T F L 87
AAGGCTGGAGATTCTGATGGTGAACGCAAGATCGGAGTTGATGAGTTCACTGCCTTGGTT 360
K A G D S D G D G K I G V D E F T A L V 107
AAGGCATATAAatgtcttctgacccaacaacacctctgtctctgaaggaaacaactttgcccac 420
K A * 109
gacctccacttttcatctactataataaatttttatactcatgatgcttatgagaagtt 480
tcctccatgcagcacactgtcaaacagatgactgtgaatgtgacgggtgtgtctctctgt 540
cttaaatatgaatttgcatactgtaaaatctgatgactctttacgggaacggacgacatac 600
gaaaagaataaatattcttttcagtataaaaaaataaataaataaataaataaataa 649
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FIGURE 1. Nucleotide and deduced amino acid sequences of the two carp parvalbumin isoforms (Cyp c 1.01 and Cyp c 1.02). Deduced amino acid sequences are given below the nucleotide sequences, and the stop codon is indicated with an asterisk. The 5' and 3' noncoding nucleotides are printed in lower case letters. The sequences were deposited in the European Molecular Biology Laboratory nucleotide sequence data base under accession numbers AJ292211 for Cyp c 1.01 and AJ292212 for Cyp c 1.02.

ants. For Cyp c 1.01, but not for Cyp c 1.02, a potential N-linked glycosylation site (aa 70–73) was predicted.

The deduced amino acid sequences were aligned with α- and β-type parvalbumin sequences deposited in the databases (Fig. 2A). Among these parvalbumins were two previously described isoforms from carp (P09227 and P02618), whose amino acid sequences had been determined by peptide sequencing of the purified proteins (9, 42). Neither Cyp c 1.01 nor Cyp c 1.02 was identical with these earlier identified carp parvalbumin isoforms, which would indicate the presence of multiple parvalbumin isoforms (14, 26).

Fig. 2A further shows that similarities between the parvalbumins from the different animal species are especially high in and around the two calcium binding regions, where most of the sequences display 100% identity. The highest sequence homologies of Cyp c 1.01 and Cyp c 1.02 were observed with β-type parvalbumins of other bony fish species (P56503 silver hake, P05941 toadfish, P02621 whiting, P05939 chub, Q91483 Atlantic salmon, P02619 pike). It was interesting to note that a β-type parvalbumin from an amphibian (P05940 from African clawed frog) showed nearly the same degree of homology (76%) and was more similar to Cyp c 1.01 and Cyp c 1.02 than parvalbumins of other bony fish species (P02620 hake with 75% identity, P02623 coelacanth with 65%



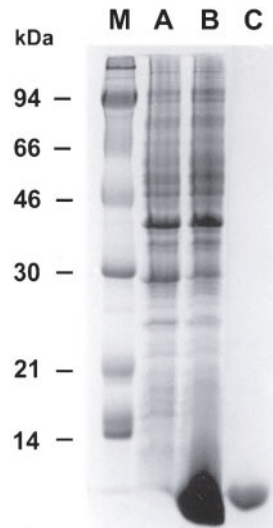


FIGURE 3. Expression and purification of recombinant carp parvalbumin, rCyp c 1.01. The Coomassie Brilliant Blue-stained SDS-PAGE containing a protein extract of host bacterium BL21 (DE3) transformed with the empty expression vector pET-17b (lane A), a protein extract of BL21 (DE3) expressing recombinant carp parvalbumin (lane B), purified recombinant parvalbumin (lane C), and a standard molecular mass marker (lane M). Molecular mass is indicated in the left margin.

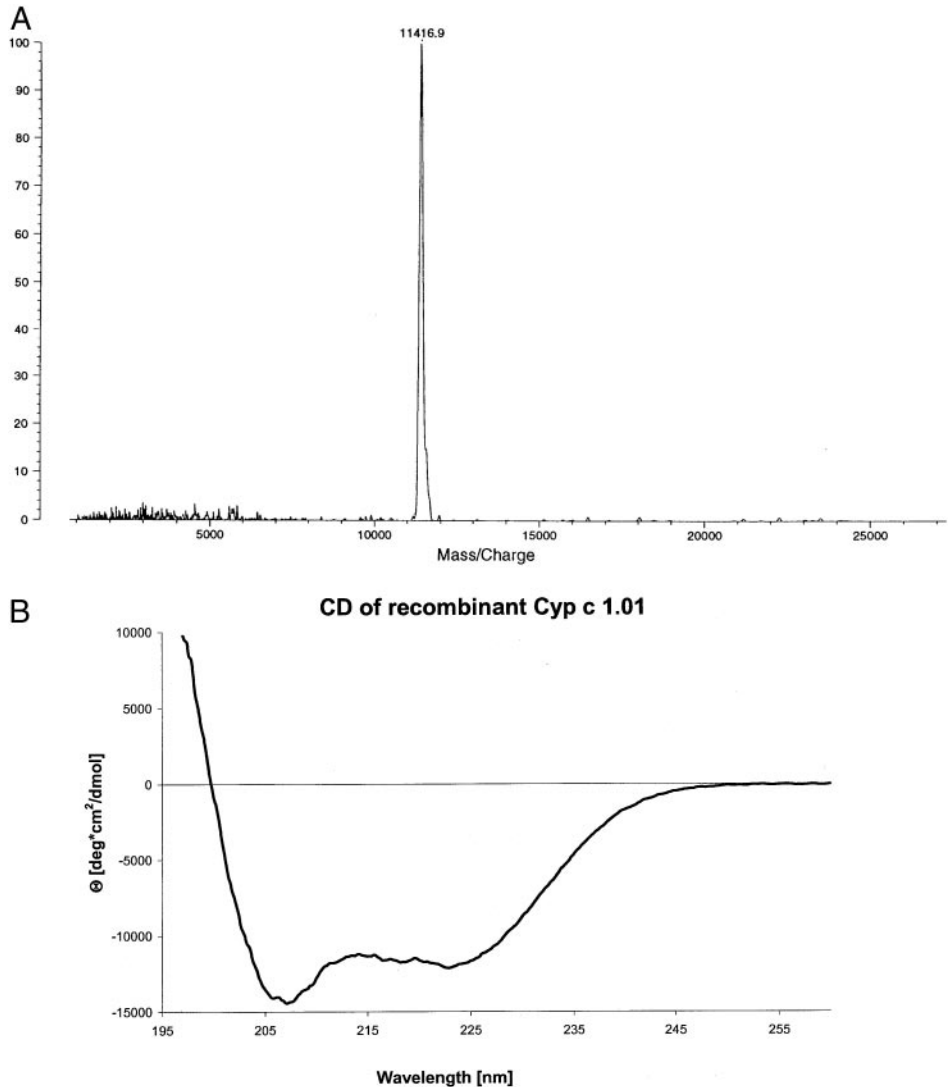
identity, and P02622 cod with 68% identity). Also, parvalbumins from a reptile (P02614 map turtle) and a bird (P19753 of chicken) exhibited significant sequence homologies of 73 and 71%, respectively, to Cyp c 1.01 and Cyp c 1.02. Even similarities to mammalian parvalbumins of the α -type were significant (59% for P80080 gerbil) and sometimes higher than the sequence identity with α -parvalbumins of cartilaginous fish species (53% for P30563 leopard shark and 50% for P02630 thornback ray).

The high sequence homology to previously identified parvalbumins allowed the construction of a three-dimensional structural model of rCyp c 1.01. The model depicted in Fig. 2B used the calcium-loaded structures of a carp parvalbumin isoform (P02618) (32) and a silver hake parvalbumin (P56503) (33) as templates. It shows the nearly spherical shape of the molecule and displays the six α -helices that are organized in three EF-hand domains, with the N-terminal nonfunctional domain forming a cap on top of the two functional Ca^{2+} binding domains. The two functional EF-hand domains are symmetrically arranged and connected through short stretches of anti-parallel β -strands.

Expression in E. coli and purification of recombinant carp parvalbumin

rCyp c 1.01 and rCyp c 1.02, which were initially expressed as β -galactosidase fusion proteins had shown comparable IgE binding capacities (data not shown). Therefore, only the cDNA coding

FIGURE 4. A, Mass spectroscopic analysis of purified recombinant carp parvalbumin. The x-axis shows the mass/charge ratio, and signal intensity is displayed on the y-axis as a percentage of the most intensive signal obtained in the investigated mass range. B, Far-UV CD analysis of purified recombinant parvalbumin. The spectrum is expressed as the mean residue ellipticity (θ) (y-axis) at a given wave length (x-axis).



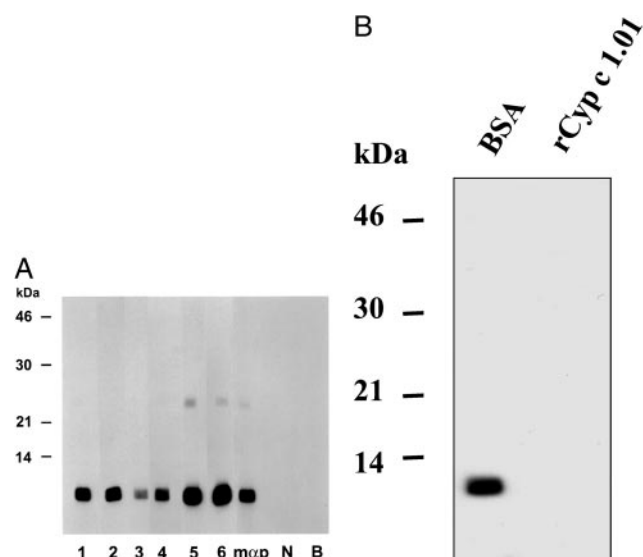


FIGURE 5. A, Ab binding capacity of recombinant carp parvalbumin. Nitrocellulose-blotted recombinant parvalbumin was exposed to sera from six fish allergic patients (lanes 1–6), to a monoclonal anti-parvalbumin Ab (lane map), to serum from a nonatopic individual (lane N), and to buffer without serum (lane B). B, rCyp c 1.01 inhibits IgE binding to natural carp parvalbumin. Nitrocellulose-blotted purified natural carp parvalbumin was exposed to serum from a fish-allergic patient that had been preincubated with recombinant carp parvalbumin (lane rCyp c 1.01) or with BSA (lane BSA). Molecular mass is indicated in the left margin.

for Cyp c 1.01 was chosen as a template for the production of recombinant carp parvalbumin as a nonfusion protein. The DNA coding for the mature Cyp c 1.01 allergen was amplified and subcloned into the expression vector pET-17b. High levels of expression of soluble rCyp c 1.01 (Fig. 3, lane B; ~30% of the total *E. coli* proteins) were obtained, and several purification steps yielded a pure, water-soluble, and folded protein of ~12 kDa (Fig. 3, lane C). The molecular mass (11,416 Da) of rCyp c 1.01 determined by mass spectroscopic analysis (Fig. 4A) was in agreement with the protein's migration in SDS-PAGE (Fig. 3) and corresponds to the molecular mass calculated for the calcium-bound form of rCyp c 1.01.

Analysis of the far-UV CD spectrum of recombinant parvalbumin showed two broad minima at 208 and 223 nm and a strong maximum <200 nm (Fig. 4B). The spectrum of the purified recombinant allergen thus resembled the features of that of purified natural carp parvalbumin (25). rCyp c 1.01 represents a folded protein containing a considerable amount of α -helical secondary structure. The latter is in accordance with the computer-aided prediction of the Cyp c 1.01 secondary structure (Fig. 2A).

rCyp c 1.01 contains most of the IgE epitopes of natural fish parvalbumins

Purified recombinant carp parvalbumin was tested for its IgE binding capacity by ELISA, dot blot, and Western blot. Fig. 5A exemplifies the IgE binding capacity of nitrocellulose-blotted rCyp c

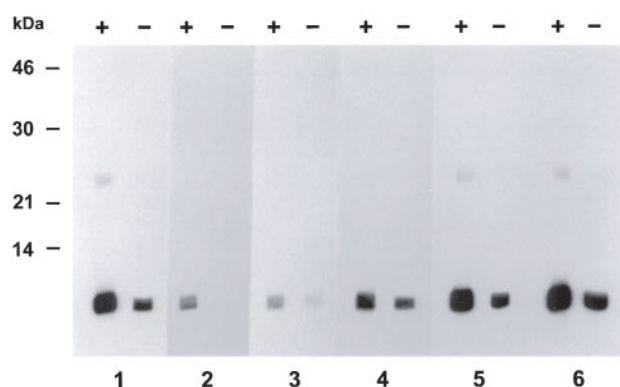


FIGURE 6. Calcium-dependent IgE recognition of recombinant carp parvalbumin. Nitrocellulose-blotted recombinant carp parvalbumin was exposed to serum IgE from six fish-allergic individuals (no. 1–6) in the presence (+) or absence (–) of calcium

1.01. Serum IgE from all six fish-allergic patients and a mAb raised against natural carp parvalbumin reacted with nitrocellulose-blotted rCyp c 1.01 (Fig. 5A). rCyp c 1.01, but not an immunologically unrelated protein (BSA), inhibited completely IgE binding to natural carp parvalbumin (Fig. 5B).

Next, we performed quantitative IgE inhibition studies to investigate whether rCyp c 1.01 contains most of the IgE binding epitopes present in protein extracts of other fish species. Sera from 16 fish-allergic patients were preadsorbed with rCyp c 1.01 or, for control purposes, with BSA, and then exposed to allergen extracts from cod, tuna, and salmon. Quantification of IgE binding by the CAP-FEIA system revealed that rCyp c 1.01 strongly inhibited IgE binding to natural fish extracts (cod, 62–96% (76% mean inhibition); tuna, 33–98% (69% mean inhibition); salmon, 41–95% (70% mean inhibition); Table I).

Calcium depletion leads to a reduction of IgE binding to rCyp c 1.01

Calcium-binding proteins can occur in their calcium-bound or calcium-depleted (apoform) forms (14). In this context it was found that several calcium-binding allergens exhibited varying IgE binding capacities depending on the presence or the absence of protein-bound calcium (24, 43). To test the influence of calcium on the IgE binding of recombinant carp parvalbumin, we exposed sera from six representative fish-allergic patients to nitrocellulose-blotted rCyp c 1.01 in the presence (+ lanes) or the absence (– lanes) of protein-bound calcium (Fig. 6). We found that calcium depletion lead to a strong reduction of IgE binding of all tested sera to rCyp c 1.01, which may be caused by a change in conformational epitopes and/or unfolding of the protein. Quantification of the IgE binding by gamma counting revealed a reduction of IgE binding to

Table II. Dependence of IgE binding to recombinant carp parvalbumin on protein-bound calcium

Patient	Percentage Reduction of IgE Binding in the Absence of Calcium (%)
1	70
2	86
3	40
4	26
5	80
6	41

Table I. Percentage inhibition of IgE reactivity to cod, tuna, and salmon extracts after preadsorption of sera with recombinant carp parvalbumin as determined by quantitative CAP-FEIA measurement

Serum	Cod (%)	Tuna (%)	Salmon (%)
n = 16	62–96 (mean 76)	33–98 (mean 69)	41–95 (mean 70)

Table III. IgG subclass and IgE responses to recombinant carp parvalbumin (rCyp c 1.01) determined by ELISA in sera from fish allergic patients and from nonatopic individuals

	rCyp c 1.01				
	IgG ₁	IgG ₂	IgG ₃	IgG ₄	IgE
Fish-allergic patients					
1	0.933	0.326	0.136	0.764	1.053
2	0.297	0.115	0.059	0.047	0.250
3	0.442	0.122	0.065	0.075	0.423
4	0.442	0.095	0.068	0.092	0.425
5	1.144	0.129	0.071	0.847	1.216
6	0.493	0.083	0.041	0.391	1.072
7	0.206	0.056	0.041	0.193	1.967
8	0.150	0.311	0.038	0.398	1.484
Nonatopic individuals					
9	0.112	0.139	0.080	0.060	0.057
10	0.146	0.154	0.124	0.072	0.057
11	0.115	0.061	0.069	0.047	0.057
12	0.098	0.065	0.038	0.037	0.054
13	0.058	0.068	0.039	0.049	0.058
14	0.094	0.078	0.053	0.075	0.057
15	0.472	0.069	0.042	0.056	0.056
16	0.077	0.071	0.032	0.037	0.056

^a Sera were diluted 1/20 for measurement of IgG subclass responses and 1/5 for measurement of IgE response. Results are displayed as mean OD values.

the apoforms ranging between 26 and 86% (57% mean reduction; Table II).

Fish-allergic patients exhibit a dissociation of IgE and IgG subclass Ab responses to rCyp c 1.01

For purified respiratory allergen molecules it has been demonstrated that allergen-specific IgG subclass responses are dissociated from IgE reactivities and also occur in nonsensitized individuals (44). Therefore, we investigated IgE and IgG₁₋₄ subclass recognition of rCyp c 1.01 as a representative food allergen using sera from eight fish allergic patients. All patients exhibited IgE reactivity to rCyp c 1.01, but IgG subclass responses varied (Table III). For example, patient 4 showed IgE and IgG₁, but no IgG₂₋₄, reactivity to rCyp c 1.01. IgE and IgG subclass recognition to rCyp c 1.01 thus showed a similar dissociation, as observed for respiratory allergens. For control purposes, we analyzed a group of grass pollen-allergic patients for IgE and IgG subclass recognition of rCyp c 1.01 and the major timothy grass pollen allergen, Phl p 5 (data not shown). Similar to that in the fish-allergic patients, we found a dissociation of IgE and IgG subclass responses. None of

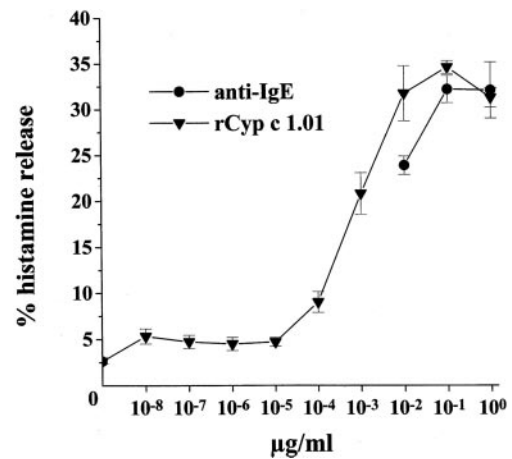


FIGURE 7. Induction of basophil histamine release with recombinant carp parvalbumin (rCyp c 1.01). Granulocytes from a fish-allergic patient were incubated with various concentrations (x-axis) of the purified recombinant protein and an anti-human IgE mAb (anti-IgE). The percentage of histamine released into the supernatant is displayed on the y-axis.

the grass pollen-allergic patients had IgE specific for rCyp c 1.01, and only those fish-allergic patients who also suffered from grass pollen allergy (patients 2 and 8) showed IgE reactivity to Phl p 5 (data not shown). However, IgG subclass responses to rCyp c 1.01 could be detected in sera of nonatopic individuals (e.g., IgG₁ reactivity of the nonatopic individual 15 to rCyp c 1.01; Table III). In summary, IgE and IgG subclass recognition of rCyp c 1.01 resembles the features observed for respiratory allergens: 1) IgE, but not IgG, subclass recognition is associated with clinical symptoms; and 2) sensitized individuals exhibit a dissociation of IgE and IgG subclass responses.

rCyp c 1.01 induces dose-dependent histamine release from basophils of fish-allergic patients

To study whether IgE recognition of rCyp c 1.01 can trigger the release of biologically active mediators from granulocytes of a fish-allergic patient, histamine release experiments were performed (Fig. 7). Purified rCyp c 1.01 induced a dose-dependent release of histamine from granulocytes of a fish-allergic patient (Fig. 7). Likewise, anti-IgE Abs induced histamine release when exposed in three concentrations to the granulocyte preparations.

Table IV. Rabbit anti-rCyp c 1.01 Abs inhibit patients' IgE binding to rCyp c 1.01^a

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13
Preimmune serum	0.720	0.191	0.315	0.417	0.536	1.697	0.234	0.248	0.306	1.015	1.763	2.215	2.500
anti-rCyp c 1.01 serum	0.072	0.124	0.172	0.176	0.264	0.069	0.049	0.052	0.057	0.053	0.122	0.075	0.110
% inhibition of IgE binding	90	35	45	58	51	96	79	79	81	95	93	97	96
Patient	14	15	16	17	18	19	20	21	22	23	24	25	
Preimmune serum	1.929	2.390	1.590	0.264	0.224	1.691	0.931	1.644	1.402	2.408	0.762	0.908	
anti-rCyp c 1.01 serum	0.070	0.096	0.084	0.064	0.240	0.057	0.044	0.072	0.060	0.164	0.083	0.137	
% inhibition of IgE binding	97	96	95	76	—	97	96	96	96	93	89	85	

^a ELISA plate-bound rCyp c 1.01 was preincubated with 1/100 diluted rabbit anti-rCyp c 1.01 Abs or with rabbit preimmune Abs and subsequently exposed to serum IgE from 25 fish-allergic patients. IgE binding was measured by ELISA. Mean OD values and percentage inhibition of IgE binding are displayed.

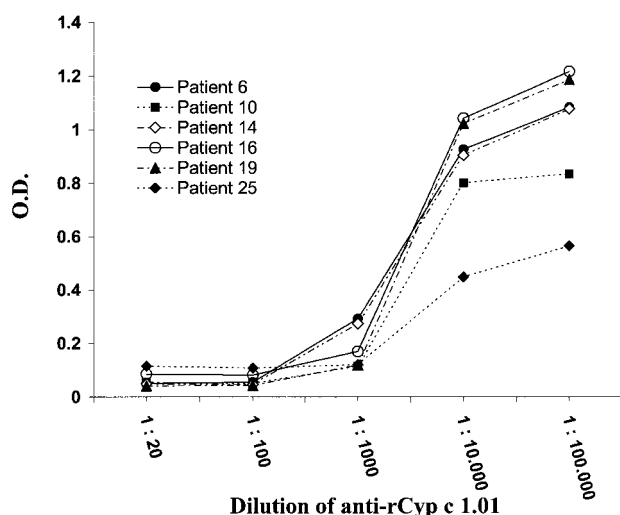


FIGURE 8. Inhibition of patients' IgE binding to rCyp c 1.01 depends on the titer of rabbit anti-rCyp c 1.01 Abs. ELISA plate-bound rCyp c 1.01 was preincubated with increasing dilutions (x-axis) of rabbit anti-rCyp c 1.01 Abs. The binding of six fish-allergic patients' IgE is displayed as the OD value on the y-axis.

Immunization with rCyp c 1.01 induces Abs that block allergic patients' IgE binding to rCyp c 1.01

To evaluate whether recombinant carp parvalbumin can induce *in vivo* protective Abs that block the binding of allergic patients' IgE to rCyp c 1.01, rabbits were immunized with the recombinant allergen. The capacity of induced anti-rCyp c 1.01 Abs to inhibit human IgE binding was examined in ELISA competition assays using sera from 25 fish-allergic patients (Table IV). For the majority of patients a strong inhibition of IgE binding, ranging between 35 and 97% (84% mean inhibition), could be observed. In the case of 14 sera, IgE binding to rCyp c 1.01 was inhibited by >90%. In only one patient (patient 18; Table IV) did anti-rCyp c 1.01 antiserum fail to inhibit IgE binding to rCyp c 1.01.

In the initial ELISA competition assays the rabbit anti-rCyp c 1.01 antiserum was diluted 1/100 to compete with allergic patients' IgE binding. Next, we investigated to what extent the rabbit anti-rCyp c 1.01 antiserum can be diluted to allow competition of allergic patients' IgE binding to rCyp c 1.01. Competition experiments were performed with sera of six fish-allergic patients using dilutions of rabbit anti-rCyp c 1.01 antiserum ranging from 1/20 to 1/100,000 (Fig. 8). Anti-rCyp c 1.01 antiserum strongly inhibited IgE binding to rCyp c 1.01 up to a dilution of 1/1000. The degree of inhibition of IgE binding was not associated with the levels of rCyp c 1.01-specific IgE Abs present in the patients' sera, because IgE binding in patients containing high (e.g., patient 16) or lower IgE levels (patient 25) was equally well inhibited by the antiserum (Fig. 8).

Discussion

Parvalbumins, which are small calcium-binding muscle proteins, represent the major and sole allergens for 95% of fish-allergic patients suffering from IgE-mediated hypersensitivity to fish. Despite their importance as major food allergens, to date no recombinant fish parvalbumin with immunological features comparable to its natural counterpart has been produced. The present study describes the isolation of two cDNAs coding for carp parvalbumin isoforms, Cyp c 1.01 and Cyp c 1.02, with comparable IgE Ab binding capacity and the expression, purification, and molecular and immunological characterization of recombinant carp parval-

bumin, rCyp c 1.01. In contrast to previous DNA-based attempts to isolate cDNAs coding for IgE-reactive parvalbumin isoforms, we used serum IgE from a fish-allergic patient for screening of a carp muscle expression library. The rCyp c 1.01 clone gave large amounts of soluble recombinant carp parvalbumin (rCyp c 1.01) when expressed in *E. coli*. Circular dichroism analysis revealed that purified rCyp c 1.01 represented a folded protein with a predominantly α -helical secondary structure comparable to that of natural carp parvalbumin.

Several experiments demonstrated that rCyp c 1.01 can be used for the diagnosis of IgE-mediated fish allergies. First, we found that immunoblotted and ELISA plate-bound rCyp c 1.01 was recognized by IgE Abs of all ($n = 60$) patients who had reacted with natural parvalbumin in carp muscle extract. Second, recombinant carp parvalbumin completely blocked IgE binding to natural carp parvalbumin in immunoblot inhibition experiments, indicating that the recombinant allergen contained most of the IgE-binding epitopes present in natural carp parvalbumin. Third, and perhaps most important, quantitative IgE inhibition studies using the CAP-FEIA system revealed that rCyp c 1.01 contained the majority (70%) of IgE epitopes present in allergen extracts of various fish species. The latter finding suggests that a single cross-reactive allergen, namely rCyp c 1.01, might represent a marker allergen to diagnose IgE-mediated cross-sensitization to various fish species. The diagnostic potential of rCyp c 1.01 was further investigated by basophil degranulation assay, which closely reflects allergic effector cell activation with the result that rCyp c 1.01 induced specific and dose-dependent histamine release from basophils of a fish-allergic patient. Biological tests (e.g., histamine and leukotriene release assays) are difficult or impossible to perform with crude fish extracts, because the presence of mediators in these extracts can cause false-positive results (45). Based on our results it may now be possible to develop rCyp c 1.01-based effector cell tests that mimic clinical symptoms better than measurements of serum IgE Abs.

rCyp c 1.01 may also be used to develop strategies for specific immunotherapy of fish allergy. Immunotherapy, the only curative approach toward type I allergy, is based on the continuous administration of increasing doses of disease-eliciting allergens, with the aim to induce a state of allergen-specific nonresponsiveness in the patient (46). Allergen-specific immunotherapy is most widely used for the treatment of respiratory and venom allergies, but is not yet established for food allergies. One possible explanation for the latter fact may be that food (e.g., fish) extracts in addition to the relevant allergens contain several ill-defined components. Our assumption that it may be possible to develop rCyp c 1.01-based molecular strategies for specific immunotherapy of fish allergy is supported by the following findings. It was demonstrated that immunization of rabbits with rCyp c 1.01 induced protective IgG Abs that inhibited the binding of patients' IgE to recombinant parvalbumin. rCyp c 1.01-induced Abs could be diluted up to 1/1000 and still block the binding of allergic patients' IgE to the allergen, suggesting that the competition of allergic patients' IgE binding to rCyp c 1.01 depended on the titer of anti-Cyp c 1.01 Abs and that the induced Abs were of high affinity. Several recent studies have rekindled interest in the concept of blocking Abs (36, 47–49). It has been demonstrated that allergen-specific IgG Abs have protective activity by suppressing allergen-induced effector cell activation and IgE-mediated presentation to T cells if they compete with the binding of allergen-specific IgE Abs. Allergen-specific IgG, which is directed to epitopes other than those defined by IgE, have no beneficial effects. Cyp c 1.01-specific Abs, probably of the latter type, could be detected in sera of fish-allergic patients as well as in individuals without fish allergy in our study. This finding

suggests that it may be important to redirect IgG responses toward IgE epitopes by appropriate vaccines. Equally, it may be necessary to modulate the ongoing Th2 response in fish-allergic patients toward a Th1 response and/or to induce tolerance at the T cell level.

Both B cell as well as T cell epitope-based therapeutic strategies will benefit from the possibility of administering high doses of allergen derivatives with reduced allergenic activity. The administration of wild-type rCyp c 1.01, even at very low doses, may carry the risk of inducing severe, life-threatening anaphylactic side effects. Therefore, it will be necessary to develop hypoallergenic rCyp c 1.01 derivatives that preserve the B cell and T cell epitopes of the wild-type allergen. Our observation that calcium depletion resulted in a greatly reduced IgE binding capacity of rCyp c 1.01 indicates that it may be possible to engineer such hypoallergenic variants of carp parvalbumin by site-directed mutagenesis of the calcium binding sites. rCyp c 1.01 derivatives may represent candidate molecules for specific immunotherapy of fish allergy with low risk of anaphylactic side effects.

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