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

Ines Swoboda,* Agnes Bugajska-Schretter, Birgit Linhart, Petra Verdinov, Walter Keller, Ulrike Schulmeister, Wolfgang R. Sperr, Peter Valent, Gabriel Peltre, Santiago Quirce, Nikolaos Douladiris, Nikolaos G. Papadopoulos, Rudolf Valenta,† and Susanne Spitzauer

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.
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 (Strategene). The three calcium-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.

![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.](http://www.jimmunol.org/)

**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.

### Table I. Clinical and serological characterization of patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Fish-Related Symptoms</th>
<th>Total IgE (kUA/L)</th>
<th>Fish-Specific IgE Binding as Determined by CAP-FEIA*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Class kUA/L</td>
</tr>
<tr>
<td>1</td>
<td>Urticaria, rhinitis, conjunctivitis</td>
<td>784</td>
<td>3.2</td>
</tr>
<tr>
<td>2</td>
<td>Rhinitis</td>
<td>1,085</td>
<td>4.8</td>
</tr>
<tr>
<td>5</td>
<td>Urticaria, rhinitis, conjunctivitis</td>
<td>826</td>
<td>9.2</td>
</tr>
<tr>
<td>7</td>
<td>Asthma, urticaria, rhinitis, conjunctivitis</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Asthma, urticaria, rhinitis</td>
<td>895</td>
<td>14.1</td>
</tr>
<tr>
<td>15</td>
<td>Rhinitis</td>
<td>183</td>
<td>11.1</td>
</tr>
<tr>
<td>22</td>
<td>Asthma, urticaria, rhinitis</td>
<td>&gt;2,000</td>
<td>8.0</td>
</tr>
<tr>
<td>25</td>
<td>Asthma, urticaria, gastrointestinal reactions</td>
<td>546</td>
<td>9.1</td>
</tr>
</tbody>
</table>

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

### Materials and Methods

#### 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 spotted in duplicates on nitrocellulose membranes (Schleicher & Schuell Microscience) and were exposed to patients’ sera diluted 1/10 in PBS (pH 7.5) containing 0.5% Tween 20. Bound IgE Abs were detected with 1/15 diluted 125I-labeled rabbit anti-human IgE Abs (RAST; Phadia), visualized by autoradiography, and quantified using a gamma counter (Wizard Automatic Gamma Counter; Wallac).

For immunoblot inhibition experiments, 5 μg/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 μg/ml per 1/10 diluted serum). Bound IgE Abs were detected with the 125I-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 radioimmunossay (Immunotech). Results are expressed as mean values of
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 μl of aliquots of four concentrations (1 μg/ml, 4 μg/ml, 16 μg/ml, and 32 μg/ml) of recombinant carp parvalbumin or Mut-CD/EF, freshly diluted from a stock solution of 100 μg/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 Abs 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 μg 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 μg/ml in 0.1 M sodium bicarbonate (pH 9.6)) and blocked with 1% (v/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 with 1/1000 diluted mouse sera (IgG1) or 1/50 diluted mouse sera (IgE) in TBST, and then with HRP-coupled goat anti-rat antiserum (Amersham Biosciences) diluted 1/1000 in TBST. The color reaction was started with 1/100 diluted mouse sera (IgG1) or 1/6 diluted mouse sera (IgE) in TBST, and then with HRP-coupled goat anti-rat antiserum (Amersham Biosciences) 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 with 1/100 diluted mouse sera (IgG1) or 1/6 diluted mouse sera (IgE) in TBST, and then with HRP-coupled goat anti-rat antiserum (Amersham Biosciences) diluted 1/1000 in TBST. The color reaction was started with 1/100 diluted mouse sera (IgG1) or 1/6 diluted mouse sera (IgE) in TBST, and then with HRP-coupled goat anti-rat antiserum (Amersham Biosciences) diluted 1/1000 in TBST. The color reaction was started with 1/100 diluted mouse sera (IgG1) or 1/6 diluted mouse sera (IgE) in TBST, and then with HRP-coupled goat anti-rat antiserum (Amersham Biosciences) diluted 1/1000 in TBST. The color reaction was started with 1/100 diluted mouse sera (IgG1) or 1/6 diluted mouse sera (IgE) in TBST, and then with HRP-coupled goat anti-rat antiserum (Amersham Biosciences) diluted 1/1000 in TBST. The color reaction was started with 1/100 diluted mouse sera (IgG1) or 1/6 diluted mouse sera (IgE) in TBST, and then with HRP-coupled goat anti-rat antiserum (Amersham Biosciences) diluted 1/1000 in TBST. The color reaction was started with 1/100 diluted mouse sera (IgG1) or 1/6 diluted mouse sera (IgE) in TBST, and then with HRP-coupled goat anti-rat antiserum (Amersham Biosciences) diluted 1/1000 in TBST. The color reaction was started with 1/100 diluted mouse sera (IgG1) or 1/6 diluted mouse sera (IgE) in TBST, and then with HRP-coupled goat anti-rat antiserum (Amersham Biosciences) diluted 1/1000 in TBST. The color reaction was started with 1/100 diluted mouse sera (IgG1) or 1/6 diluted mouse sera (IgE) in TBST, and then with HRP-coupled goat anti-rat antiserum (Amersham Biosciences) diluted 1/1000 in TBST. The color reaction was started with 1/100 diluted mouse sera (IgG1) or 1/6 diluted mouse sera (IgE) in TBST, and then with HRP-coupled goat antisa...
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-CD, 3.1–93.5% reduction of the IgE reactivity; Mut-CD/EF, 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-CD/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 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 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 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 who was titrated for IgE reactivity to the three parvalbumin mutants. The percentage of total histamine released into the supernatant is displayed on the y-axis.

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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Wild-Type</th>
<th>Mut-EF</th>
<th>Mut-CD</th>
<th>Mut-CD/EF</th>
<th>Percent Wild Type</th>
</tr>
</thead>
<tbody>
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<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
<td></td>
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<tr>
<td>1</td>
<td>151.0</td>
<td>39.9</td>
<td>21.3</td>
<td>1.2</td>
<td>0.45</td>
</tr>
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<td>2</td>
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<td>1.2</td>
<td>0.56</td>
</tr>
<tr>
<td>3</td>
<td>211.2</td>
<td>69.9</td>
<td>39.9</td>
<td>1.2</td>
<td>0.45</td>
</tr>
<tr>
<td>4</td>
<td>151.0</td>
<td>39.9</td>
<td>21.3</td>
<td>1.2</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* 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).
when applied at the highest concentration (32 μg/ml; Fig. 5). In contrast, Mut-CD/EF induced no skin reactions even though Mut-CD/EF induced specific IgG1 Abs, which recognized Mut-CD/EF. ELISA analyses revealed that in each of the immunized mice Mut-CD/EF, BALB/c mice were immunized with purified Mut-CD/EF. Abs that cross-react with parvalbumins from important fish species, 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 IgG 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 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 Cyp c 1 mutant may be considered as an approach for mucosal application (reviewed in Ref. 43) or conjugated to immunostimulatory sequences (Ref. 44 and reviewed in Ref. 42). Furthermore, it will also be possible to administer the Mut-CD/EF derivative by skin prick testing in fish allergic Greek children. In Proceedings of the EAACI 2006 Congress, June 10–14, European Academy of Allergology and Immunology, Stockholm, Sweden, p. 22.

References


