IgE-binding epitopes of enolases, a class of highly conserved fungal allergens

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Background: ***Cladosporium herbarum*** **and** ***Alternaria alternata*** **are two of the most prominent fungal species inducing type I allergy. Previously, we have demonstrated that enolase (Cla h 6) is the second most important allergen of** ***C herbarum*** **in terms of frequency of sensitization.**
Objective: IgE-reactive B-cell epitopes of ***C herbarum*** **enolase were analyzed, and cross-reactivity between fungal enolases was investigated.**
Methods: Cla h 6 glutathione-S-transferase fusion peptides were constructed by means of PCR cloning. ***A alternata*** **enolase (Alt a 5) was isolated by screening a complementary (c)DNA expression library with a** ***C herbarum*** **enolase DNA probe.**
Results: Mapping of Cla h 6 IgE-binding epitopes identified a peptide with a length of 69 amino acids (peptide 9), which bound IgE from 8 of 8 patients. Analysis of the conformation of peptide 9 revealed that it does not form a compact structure but rather spans the whole length of the protein, with side chains exposed to solvent at 3 locations. Peptide 9 in the context of ***Escherichia coli*** **glutathione-S-transferase not only binds IgE but also competitively inhibits IgE binding to Alt a 5. This result indicates that the epitope or epitopes on peptide 9 constitute a major cross-reacting epitope or epitopes on the enolases from** ***C herbarum*** **and** ***A alternata*** **in the case of the one patient tested.**
Conclusions: We demonstrated that the glycolytic enzyme enolase is an allergen not only in ***C herbarum*** **but also in** ***A alternata*****. Additionally, enolase was shown to exhibit high cross-reactivity to other fungal enolases. On the basis of the results presented here, we propose the use of recombinant Cla h 6 or maybe even peptide 9 of Cla h 6 for diagnosis and possibly therapy of mold allergy. (J Allergy Clin Immunol 2000;106: 887-95.)**

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Enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) is a ubiquitous enzyme that catalyzes the interconversion of 2-phospho-D-glycerate and phosphoenolpyruvate in glycolysis. The enzyme is phylogenetically highly conserved with respect to the amino acid sequence.[1] It has been isolated and characterized from various sources, ranging from bacteria to higher vertebrates.[2-4]

In addition to its essential role in glycolysis, enolase has other important functions. Enolase was identified as a lens structural protein, named τ-crystallin, in various vertebrates.[5] An isoprotein of *Saccharomyces cerevisiae* enolase was shown to be the heat shock protein HSP48. This isoprotein of *S cerevisiae* enolase seems to be important for thermal tolerance and growth control.[6] In *Candida albicans*[7] and *S cerevisiae*[8] enolase has been found in association with the cell wall.

Enolase was first identified as an allergen in *S cerevisiae*.[9-11] Subsequently, we showed that enolase is an allergen (Cla h 6) in *Cladosporium herbarum*. Recombinant (r)Cla h 6 was produced in *Escherichia coli* and analyzed for its IgE-binding activity.[12,13]

Here we present the identification of IgE-binding epitopes of *C herbarum* enolase. We also show that *Alternaria alternata* enolase is an important allergen of this mold species. Furthermore, IgE cross-reactivity between the enolases of *C herbarum*, *Alternaria alternata*, *C albicans*, and *Aspergillus fumigatus* is demonstrated.

METHODS

Patients and sera

Patients allergic to *C herbarum* and *A alternata* were selected according to typical case history, positive skin prick test response, and RAST (Pharmacia-Upjohn, Uppsala, Sweden) class greater than 3. Sera were stored at –20°C.

Mold strains, growth conditions, and fungal extracts

A type strain of *A fumigatus* (MA 148, IFG 0502, Prague 1945) was a gift from H. J. Prillinger (University of Agriculture, Department of Applied Microbiology, Vienna, Austria). Cultivation and protein extraction of *C herbarum*, *A alternata*, and *A fumigatus*

Abbreviations used
DOC: Deoxycholate
GST: Glutathione-S-transferase
rnf: Recombinant nonfusion

were done, as described previously.[12] *S cerevisiae* was cultivated in yeast peptone dextrose overnight at 28°C. Cells from a 5-mL culture were harvested and resuspended in 0.3 mL of 10 mmol/L TRIS-HCl plus 1 mmol/L EDTA (pH 8.0). Cell disruption was carried out by adding 0.5 mL of 0.45-mm-diameter glass beads (Braun, Melsungen, Germany) followed by shaking in a Braun homogenizer. The homogenate was centrifuged at 9000*g* at 4°C for 15 minutes, and the clear supernatant was used for further experiments.

Screening of an *A alternata* Uni-Zap XR expression complementary DNA library

Construction of an *A alternata* complementary (c)DNA expression library in the vector Uni-Zap XR by using the ZAP-cDNA synthesis kit of Stratagene (La Jolla, Calif) was described previously.[12] The library (in its excised phagemid form) was screened by means of hybridization with a digoxigenin-labeled (Boehringer, Mannheim, Germany) *C herbarum* enolase DNA probe (GenBank Accession Number X78226).[12] Digoxigenin labeling was performed by using the ECL direct nucleic acid labeling and detection system of Amersham-Pharmacia-Biotech (Buckinghamshire, UK).

DNA sequencing

The sequences of all constructs were determined by means of cycle sequencing with an ABI-PRISM Cycle Sequencing Kit and the ABI-Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, Calif).

PCR cloning

B-cell epitopes of Cla h 6 were analyzed by applying a PCR strategy (Fig 1) with 8 oligonucleotide primers. The forward primers (1-4) were designed to contain a *Bam*HI site or *Bam*HI-*Nde*I site (in the case of peptide 1), respectively. Reverse primers (5-8) were equipped with an *Eco*RI site. The numbers in parentheses refer to the nucleotide numbering of the *C herbarum* enolase coding sequence: primer 1 (1-21 bp) 5'GAG**GGATCCCATATG***CCTATCTCCAAGATCCAC*3'; primer 2 (361-378 bp) 5'GAG**GGATCC***GCTGCCGCCGCCGAGAAG*3'; primer 3 (742-759 bp) 5'GAG**GGATCC***GCCTCCTCCGAGTTCTAC*3'; primer 4 (1120-1137 bp) 5'GAG**GGATCC***ATGGTCTCTCACCGTTCC*3'; primer 5 (178-195 bp) 5'GA**GAATTC***AGCCTTGGTCACACCCTT*3'; primer 6 (549-566 bp) 5'GA**GAATTC***ACCTCAGCACCCTGGCGC*3'; primer 7 (931-948 bp) 5'GA**GAATTC***GCCAGAGGTCTTGTAGAA*3'; and primer 8 (1306-1323 bp) 5'GA**GAATTC***TTACAAGTTGATGGCAGT*3'. Linker nucleotides are indicated in bold, and nucleotides of the Cla h 6 coding sequence are shown in italics. The *Nde*I site is underlined. Ten PCR reactions were performed by using the Cla h 6 cDNA[12] as a template. The resulting constructs were designated as follows: glutathione-S-transferase (GST)-rCla h 6 (1-441, peptide 1); GST-rCla h 6 (1-316, peptide 2); GST-rCla h 6 (1-189, peptide 3); GST-rCla h 6 (1-65, peptide 4); GST-rCla h 6 (120-441, peptide 5); GST-rCla h 6 (247-441, peptide 6); GST-rCla h 6 (373-441, peptide 7); GST-rCla h 6 (120-316, peptide 8); GST-rCla h 6 (120-189, peptide 9); and GST-rCla h 6 (247-316, peptide 10). PCR reactions were performed with the Pfu-polymerase (Stratagene) according to the manufacturer's instructions. The PCR products were digested and ligated into the polycloning sites of the vector pGEX-2T (Amersham-Pharmacia-Biotech).

Expression and purification of enolases

Enolase-coding sequences were cloned into pMW172[14] or pGEX-2T (Amersham-Pharmacia-Biotech) expression vectors and transformed into *E coli* strain *BL21* (DE3) (Stratagene). Expression of the respective constructs was performed as described previously.[12] *E coli* lysates of the recombinant proteins were analyzed by using SDS-PAGE,[15] Coomassie staining, and IgE immunoblots.[12]

E coli cells were lysed at room temperature for 30 minutes by adding 0.47 mg of lysozyme. To digest genomic DNA, DNase I from Sigma-Aldrich (Vienna, Austria) was added to a final concentration of 6 μg/mL, and the mixture was incubated for 15 minutes at room temperature. After centrifugation at 10,000*g*, the pellet was used for purification of inclusion bodies. First, pellets were resuspended in 4.5 mL of deoxycholate (DOC) solution (20 mmol/L TRIS-HCl [pH 8.0], 200 mmol/L NaCl, 1% [wt/vol] deoxycholate, and 2 mmol/L EGTA) and then incubated for 30 minutes at room temperature. After centrifugation at 10,000*g*, pellets were resuspended in 7 mL of diluted DOC solution (1 volume of DOC solution and 2 volumes of distilled water) and centrifuged (10,000*g*) for 5 minutes. This step was repeated 4 times. For removal of the DOC solution, pellets were washed twice in 3 mL of 10 mmol/L TRIS-HCl (pH 8.0) and 3% (vol/vol) 2-propanol and centrifuged (10,000*g*) for 5 minutes. For solubilization of the inclusion bodies, pellets were resuspended in 3 mL of urea solution (8 mol/L urea, 10 mmol/L TRIS-HCl [pH 8.0], and 1 mmol/L EGTA) and centrifuged. An aliquot of the remaining pellets and supernatants was analyzed by SDS-PAGE followed by Coomassie staining. For renaturation of the recombinant proteins, 8 mol/L urea solutions were dialyzed stepwise against decreasing concentrations of urea. The first dialysis step was performed overnight against 6 mol/L urea, 10 mmol/L TRIS-HCl (pH 8.0), and 1 mmol/L EDTA. In the subsequent steps urea concentrations were 4, 2, 1, and 0 mol/L, resulting in complete solubilization of protein. Specific enzymatic activity of full-length enolase was determined as an indicator of a native-like folding of the recombinant protein.

Enzymatic activities of enolases from *C herbarum* and *A alternata*

For enzyme assays, 0.5 μg of enolase was added to 1 mL of assay mixture (20 mmol/L Na_2HPO_4 [pH 7.4], 400 mmol/L KCl, 0.01 mmol/L EDTA, 2 mmol/L $MgCl_2$, and 2 mmol/L 2-phospho-D-glycerate). The enzyme activity was determined at room temperature by transferring the mixture into a quartz cuvette (d = 0.5 cm) and measuring the absorption at a wavelength of 240 nm for 3 minutes.[16]

IgE cross-reactivity between enolases of different molds

Fifty micrograms of purified recombinant nonfusion (rnf) protein (eg, rnfCla h 6) was separated on a preparative SDS-PAGE,[15] electroblotted onto Immobilon-P membrane (Millipore, Bedford, Mass),[17] and subsequently stained with Ponceau-Red.[18] The protein band was cut out with a scalpel and incubated with 0.7 mL of 10-fold diluted serum from a patient allergic to *C herbarum* and *A alternata*. The preincubated serum was loaded onto an Immobilon-P membrane strip containing electrophoretically separated and subsequently blotted fungal extract (10 μg) or recombinant enolase (1 μg of purified rnfAlt a 5 or rnfCla h 6). Detection of specifically bound IgE antibodies was performed as described.[12]

Purification and detection of GST fusion peptides

Purification by glutathione-sepharose-4B chromatography and immunodetection with goat anti-GST antibody (Amersham-Phar-

macia-Biotech) of GST fusion proteins was done according to the manufacturer's recommendations. Subsequently, purified fusion proteins were tested in IgE immunoblots[12] by using sera from patients allergic to mold.

Site-directed mutagenesis of rCla h 6 and rCla h 6 (120-189, peptide 9)

The pMW172/rnfCla h 6 plasmid was used to introduce 2 different mutations by using the QuickChange Site Directed Mutagenesis Kit (Stratagene). In the first construct, rCla h 6 (K141A), lysine 141 was replaced by alanine; in the second construct, rCla h 6 (K141Q), lysine 141 was replaced by glutamine.

The fragments of rCla h 6 corresponding to peptide 9 but bearing the mutations K141A and K141Q, respectively, were subcloned into pGEX-2T (through *Bam*HI and *Eco*RI), resulting in the peptides GST-rCla h 6 (120-189, K141A) and GST-rCla h 6 (120-189, K141Q).

Molecular modeling of *C herbarum* enolase

The modeling of the *C herbarum* enolase structure was done on the basis of sequence alignment by using a homology-based approach. Comparison of the crystal structure of *S cerevisiae* enolase[19,20] with the structure of *C herbarum* enolase revealed differences in the loop regions. Modification of the loop regions was therefore required and was performed manually by using the TURBO-FRODO molecular modeling program.[21] Subsequently, the structure of *C herbarum* enolase was refined by using the ICM program.[22]

RESULTS

Epitope mapping of *C herbarum* enolase

IgE reactivity of patients' sera with *C herbarum* extract is shown in Fig 2, *A*.

Mapping of IgE-binding epitopes of *Cl herbarum* enolase was performed by immunoblotting of 10 fusion peptides produced by PCR cloning. Ten recombinant peptides (Fig 1) were fused to the C-terminus of *E coli* glutathione-S-transferase by using genetic engineering, expressed in *E coli*, and purified by means of affinity chromatography (Fig 2, *B*). These 10 fusion peptides were tested in standard immunoblots (see the "Methods" section) for IgE reactivity by using sera from 10 *C herbarum*–sensitized patients. Six peptides (peptides 1, 2, 3, 5, 8, and 9) showed specific IgE reactivity with sera from all those patients, which reacted with rnfCla h 6. Peptides 4 and 6 showed IgE-binding activity for some, but not for all, patients tested (indicating minor epitopes). Two peptides (peptides 7 and 10) displayed no IgE reactivity at all (Fig 2, *C*). In a control experiment sera from patients allergic to *C herbarum* did not show any specific IgE binding to glutathione-S-transferase (Fig 2, *D*). Additional controls (not included in Fig 2) showed that none of the peptides reacted to nonatopic or atopic but non-mold-specific human serum.

A peptide 69 amino acids in length, peptide 9 (rCla h 6 [120-189]), showed IgE reactivity with all patients reactive to rnfCla h 6. Therefore rCla h 6 (120-189) seems to contain one or more immunodominant B-cell epitope or epitopes of *C herbarum* enolase. Peptide 9 corresponds to the overlapping region of 5 larger IgE-binding peptides (Fig 1). Patients 8 and 9 displayed little

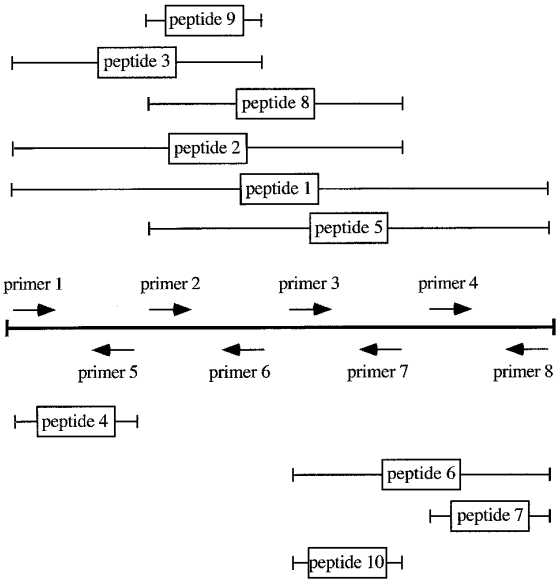

FIG 1. Ten *C herbarum* enolase peptides were constructed by combining 8 primers in 10 PCR reactions. For peptide 1, which represents the full-length enolase, primers 1 and 8 were combined (peptide 2: primers 1 + 7; peptide 3: primers 1 + 6; peptide 4: primers 1 + 5; peptide 5: primers 2 + 8; peptide 6: primers 3 + 8; peptide 7: primers 4 + 8; peptide 8: primers 2 + 7; peptide 9: primers 2 + 6; and peptide 10: primers 3 + 7). IgE-reactive peptides (peptides 1, 2, 3, 5, 8, and 9) are displayed at the *top,* whereas the other fragments shown at the *bottom* of the figure were either weakly reactive (peptides 4 and 6) or nonreactive (peptides 7 and 10) with IgE.

or no IgE binding to natural *C herbarum* enolase (Fig 2, *A*), as well as to recombinant full-length GST-Cla h 6 (Fig 2, *C*) and GST-peptide 9.

To evaluate whether peptide 9 represents a cross-reactive B-cell epitope, we performed immunocompetition experiments with full-length Cla h 6 and Alt a 5. Using peptide 9–depleted serum, a significant reduction in IgE reactivity to enolases of *C herbarum* and *A alternata* (Fig 3) was observed when compared with nondepleted serum. These results suggest that for the patient tested, the region corresponding to peptide 9 constitutes a major IgE-binding epitope not only in *C herbarum* enolase but also in the closely related *A alternata* enolase.

Molecular modeling of *C herbarum* enolase

In an attempt to understand the results of our epitope mapping in terms of the 3-dimensional structure of Cla h 6, we undertook modeling of this structure on the basis of the crystal structure of *S cerevisiae* enolase 1.[19,20] The amino acid sequence of Cla h 6 has 74% identity to enolase 1 from *S cerevisiae*. This opens the possibility of simulating the 3-dimensional structure of the *C herbarum* enolase by using a homology-based modeling approach.

The protein sequence alignment of *S cerevisiae* enolase 1[23] and Cla h 6[12] revealed that there are only 3 sites of one or 2 residue deletions or insertions (not shown). In the 3-dimensional structure of *S cerevisiae* enolase, they corre-

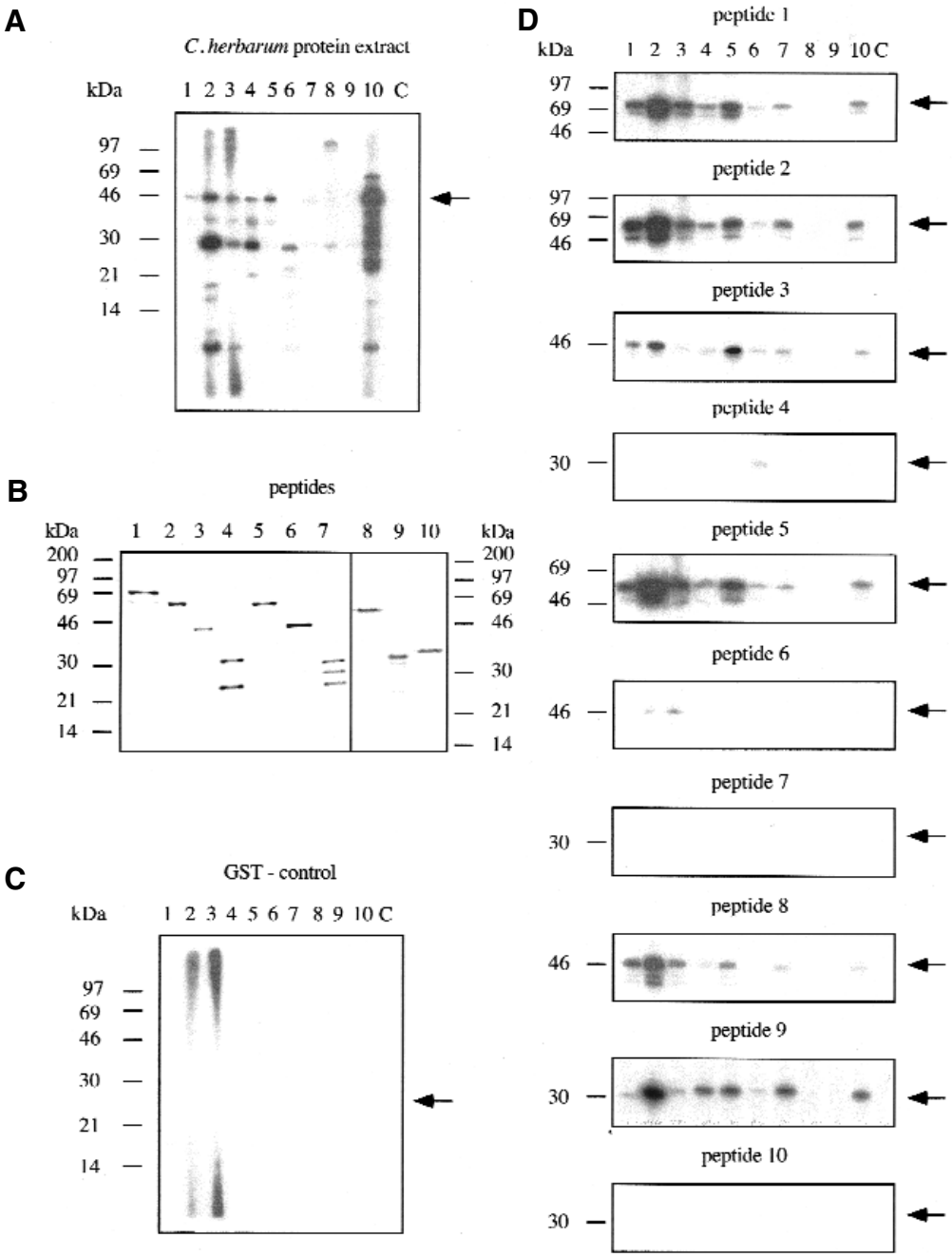

FIG 2. A, IgE blot of *C herbarum* extract tested with sera from 10 different patients with mold allergy *(lanes 1-10).* In *lane C* the extract or the given peptide was solely incubated with iodine 125–labeled rabbit anti-human IgE antibodies. The *arrow* indicates the electrophoretic mobility of Cla h 6. **B,** Coomassie-stained SDS-PAGE of 10 Cla h 6 peptides fused to GST. **C,** IgE reactivities of rCla h 6 (1-441) (peptide 1), rCla h 6 (1-316) (peptide 2), rCla h 6 (1-189) (peptide 3), rCla h 6 (1-65) (peptide 4), rCla h 6 (120-441) (peptide 5), rCla h 6 (247-441) (peptide 6), rCla h 6 (373-441) (peptide 7), rCla h 6 (120-316) (peptide 8), rCla h 6 (120-189) (peptide 9), and rCla h 6 (247-316) (peptide 10) were tested with sera from 10 patients with mold allergy. The *arrows* indicate the electrophoretic mobility of the fusion peptides. **D,** GST *(arrow)* was tested with patients' sera for nonspecific reactivity.

spond to the loop regions. The conformation of these loops should be different in the *C herbarum* enolase structure. All 3 loops were modified manually, as described briefly in the "Methods" section. Then the structure of *C herbarum* enolase was modeled and refined.

The molecular modeling revealed that the following residues of the 120-189 fragment are exposed to the solvent and therefore may interact with IgE: Ala124-Leu130, Asp136-Pro143, Ser158-Arg163, Gly175, Pro177, Thr180, Arg184, and Glu188 (Fig 4). Further analysis, taking into

consideration the size of the antigen-binding domain of IgE, suggests that Ala124-Leu130, Asp136-Pro143, and Arg163 are the most probable regions for IgE binding.

In vitro site-directed mutagenesis of Cla h 6 peptide 9

To get more insights into the IgE-binding properties of rCla h 6 (120-189), we performed in vitro mutagenesis using a PCR-based approach. By using this method, Lys141 was mutated to Gln141, as well as to Ala141. Amino acid Lys141 was chosen for mutagenesis because modeling of the structure of *C herbarum* enolase (Fig 4) showed that within peptide 9, 2 lysine residues (positions 141 and 142) are exposed to the surface of the molecule. We assumed that Lys141 is located in a critical position for the binding of IgE antibodies because exposed lysine or arginine residues have been found to be essential for antigen-antibody interactions.[24]

The 2 mutations K141A and K141Q were introduced in the context of rnfCla h 6, resulting in the construct rnfCla h 6 (K141A) and rnfCla h 6 (K141Q), respectively. Starting from these 2 constructs, the fragments corresponding to mutated peptide 9 of *C herbarum* enolase were subcloned into the expression vector pGEX-2T, resulting in mutated GST-fusion peptides. These constructs were tested for IgE reactivity with the patient's serum. Comparison of wild-type rnfCla h 6 with rnfCla h 6 (K141A) and rnfCla h 6 (K141Q) mutant proteins revealed that mutations K141A and K141Q did not change the overall IgE-binding characteristics of full-length rnfCla h 6. This could be due to other (unchanged) IgE epitopes on Cla h 6. Therefore we investigated the IgE-binding properties of the in vitro mutagenized peptides of 69 amino acids in length. Our results showed that the IgE reactivity of GST-rCla h 6 (120-189, K141A) and GST-rCla h 6 (K141Q) was comparable to the GST peptide 9 of wild-type sequence (data not shown). Therefore the predicted IgE-binding epitope (136-143) of peptide 9 is probably ruled out as a dominant epitope, but the 2 other predicted epitopes, which are formed by amino acids (124-130) and by Arg163, could still be major IgE-binding sites.

Cloning and sequence analysis of *A alternata* enolase

A alternata enolase was isolated by hybridization screening with a Cla h 6 DNA probe of a Uni-ZAP XR cDNA expression library[12] and designated Alt a 5 in accordance with the World Health Organization/International Union of Immunologic Societies allergen nomenclature system.[25*] The open reading frame encoding the enolase spans a length of 1317 bp (438 amino acids), corresponding to a protein of 46.9 kd. The amino acid sequences of *A alternata* enolase and *C herbarum* enolase are 89% identical. The sequence identities of Alt a 5 with *S cerevisiae* enolase and *C albicans* enolase are 73%.

*The nucleotide sequence of *A alternata* enolase has been submitted to the GenBank database under GenBank Accession Number U82437. The abbreviation Alt a 5 for *A alternata* enolase has been deposited at the WHO/IUIS Allergen Nomenclature Sub-Committee.

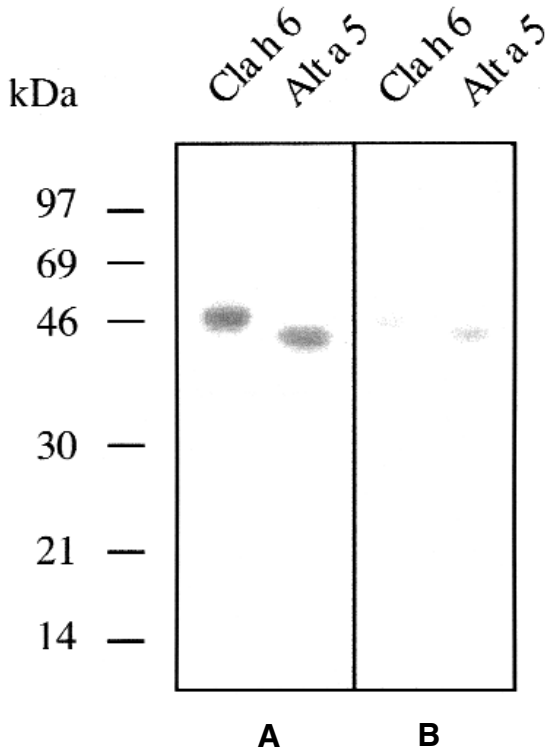

FIG 3. Cross-reactivity between peptide 9 and full-length enolases of *C herbarum* and *A alternata*. **A,** One microgram of rnfCla h 6 and rnfAlt a 5 was separated by using SDS-PAGE transferred to Immobilon-P membrane (Western blot) and incubated with non-depleted serum of a single patient. **B,** Western blot containing 1 μg of rnfCla h 6 and rnfAlt a 5 was incubated with peptide 9–depleted patient serum. In both cases (**A** and **B**) the same amount of serum was used, and both immunoblots were exposed for the same amount of time.

IgE binding to fungal enolases

The importance of *A alternata* enolase as an allergen was investigated by testing 23 patients' sera that reacted positively with protein extract of *A alternata* for IgE reactivity with the recombinant protein. For this purpose, *A alternata* enolase was produced as a nonfusion protein in *E coli* (rnfAlt a 5) purified to apparent homogeneity from bacterial lysates and tested for enzymatic activity (specific activity of rnfCla h 6 and rnfAlt a 5 was 25.8 U/mg × minutes and 20.9 U/mg × minutes, respectively). Purified rnfAlt a 5 was recognized by 22% (n = 23) of *A alternata*-sensitized patient sera. Within the patients tested, Alt a 5 was the second most important of 5 recombinant nonfusion allergens (Alt a 1[26]; Alt a 5; Alt a 7, the YCP4-homolog protein[12]; Alt a 10, the aldehyde dehydrogenase[12]; and Alt a 6, the acidic ribosomal protein P2[12]) in terms of frequency of sensitization.

Cross-reactivity between recombinant *C herbarum* and *A alternata* enolases was assessed by using IgE-inhibition experiments. All immunoblots shown in Figs 3 and 5-7 were performed with serum from one patient allergic to mold who was sensitized to enolase. Membrane strips

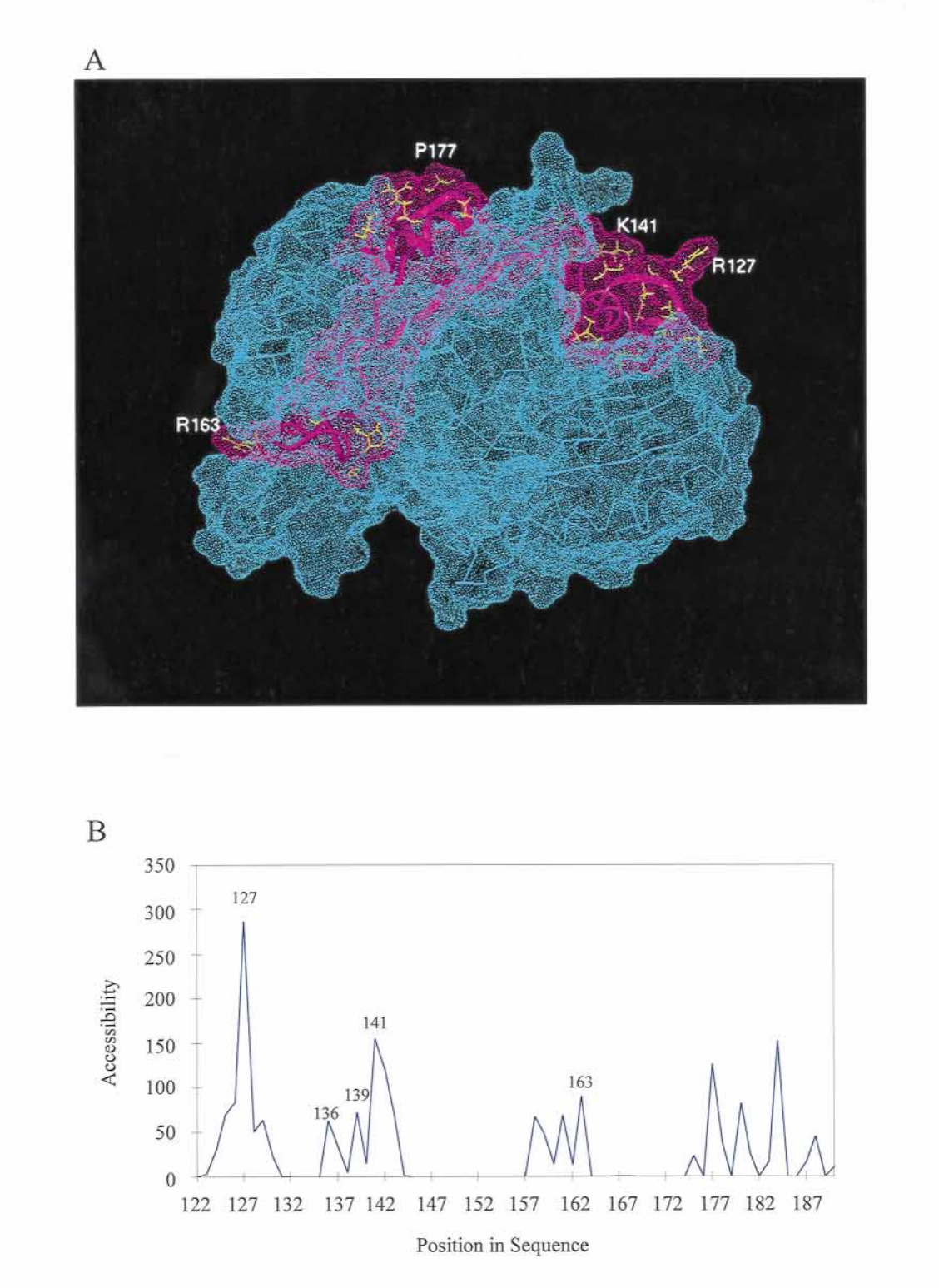

FIG 4. A, The ribbon representation of peptide 9, the 69-residue fragment that binds IgE antibodies from allergic patients, is displayed in magenta. The side chains predicted to interact with IgE are shown in yellow. **B,** The exposed surface for each residue of peptide 9 in the modeled structure is plotted over the residue position. The values of the side chain accessibility were calculated by using the TURBO-FRODO program package,[21] according to the method of Lee and Richards,[31] with a probe size of 1.4 Å.

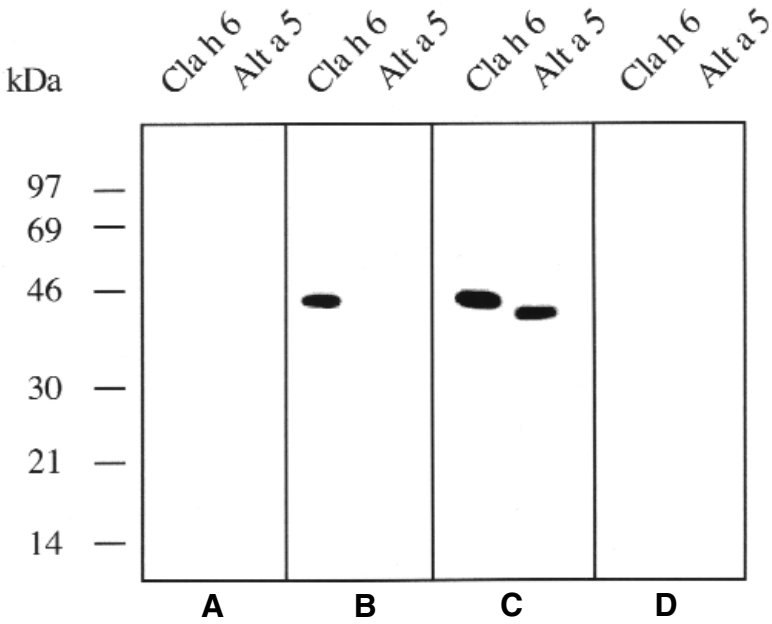

FIG 5. Cross-reactivity between *C herbarum* and *A alternata* enolases was investigated. **A,** The patient's serum was preincubated with purified rnfCla h 6 (blotted to an Immobilon-P membrane) and afterward loaded onto membrane strips containing blotted rnfCla h 6 or rnfAlt a 5. **B,** rnfAlt a 5–depleted serum was incubated with rnfCla h 6 or rnfAlt a 5. **C,** rnfCla h 6 and rnfAlt a 5 were incubated with nondepleted patient's serum. **D,** rnfCla h 6 and rnfAlt a 5 were solely incubated with iodine 125–labeled rabbit anti-human IgE.

containing purified rnfClah6 or rnfAlt a 5 were preincubated with serum for complete depletion of specific IgE antibodies. When rnfCla h 6–depleted serum was tested with rnfAlt a 5, no IgE reactivity was observed, indicating that all IgE-binding epitopes of rnfAlt a 5 are also present on rnfCla h 6 (Fig 5, *A*). However, rnfAlt a 5–depleted serum still showed residual IgE binding to rnfCla h 6 (Fig 5, *B*), suggesting that Cla h 6 contains additional IgE-binding epitopes when compared with Alt a 5. In control experiments rnfAlt a 5–depleted serum was used to visualize membrane-bound rnfAlt a 5 (Fig 5, *B*). No rnfAlt a 5–specific IgE antibodies were detected, showing that the depletion was complete. Nondepleted serum contained IgE antibodies specific for rnfCla h 6 and rnfAlt a 5 (Fig 5, *C*). No signals were observed when only the secondary antibody was used (Fig 5, *D*). The experiments shown in Fig 5 were repeated with sera from 3 other patients with mold allergy, with essentially the same results.

Because our experiments demonstrated that Cla h 6 contains additional IgE-binding epitopes compared with Alt a 5, we used purified rnfCla h 6 in inhibition experiments to evaluate whether enolase represents a cross-reactive allergen in other molds. IgE immunoblots of extracts from *S cerevisiae*, *C albicans*, and *A fumigatus* (Fig 6, *B*) showed a prominent IgE-reactive protein band with a molecular mass between 46 and 48 kd. Preincubation of patient serum with rnfCla h 6 completely abolished or reduced IgE binding to proteins of similar weight in extracts of *A fumigatus* and *C albicans* (Fig 7, *A*). Taken together, our results show that enolase is a cross-reactive allergen in *C herbarum*, *A alternata*, *S cerevisiae*, *C albicans*, and *A fumigatus*. These results clearly indicate that not only the sequences of these fungal enolases but also their IgE-binding structures are highly homologous.

DISCUSSION

In this study we performed epitope mapping of *C herbarum* enolase, which is an important fungal allergen. Identification of IgE-binding epitopes is the first step toward a better understanding of the molecular features of an allergen and can help to devise new therapeutic approaches.

At the present time, we (this work) and others[27-30] have to rely on indirect methods to determine IgE-binding epitopes on allergens. In our view the expression of allergenic fragments in the context of a neutral (non-IgE-binding) carrier protein is presently the method of choice. Our results show that a relatively short part of enolase (amino acids 120-189) is capable of binding IgE from 8 of 8 patients. However, the carrier protein GST is still needed for this interaction, although it does not bind patients' IgE by itself. The 69 amino acids (120-189) from *C herbarum* enolase, termed peptide 9, were also expressed in *E coli* as a nonfusion polypeptide but did not bind IgE. To clarify this apparent discrepancy, we investigated the solution structure of highly purified his-tagged nonfusion peptide 9 by using circular dichroism spectroscopy. The result indicates that nonfusion peptide 9, which is very extended in the context of the complete enolase structure, may adopt a new structure in solution. In our view this is a possible explanation for the

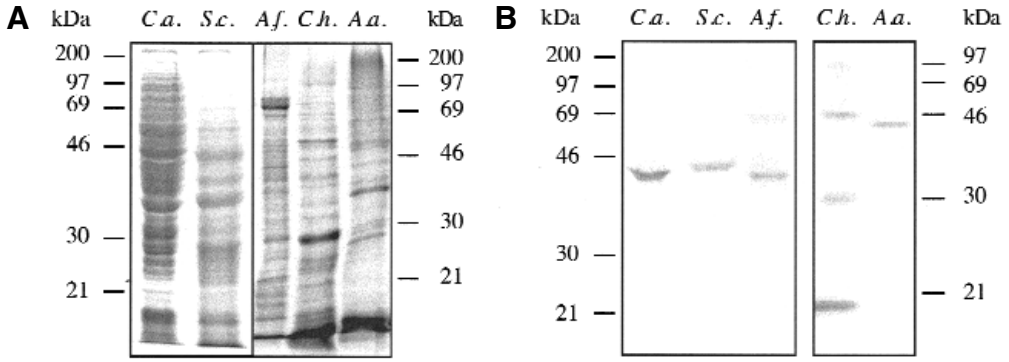

FIG 6. A, Coomassie-stained SDS-PAGE of extracts of *C albicans (C.a.)*, *S cerevisiae (S.c.)*, *A fumigatus (A.f.)*, *C herbarum (C.h.)*, and *A alternata (A.a.)*. **B,** IgE blot of mold extracts shown in panel **A**. Mold extract corresponding to 10 μg of protein was loaded onto an SDS-PAGE, electroblotted, and tested with the patient's serum for IgE reactivity. The prominent band around 46 kd corresponds to enolase.

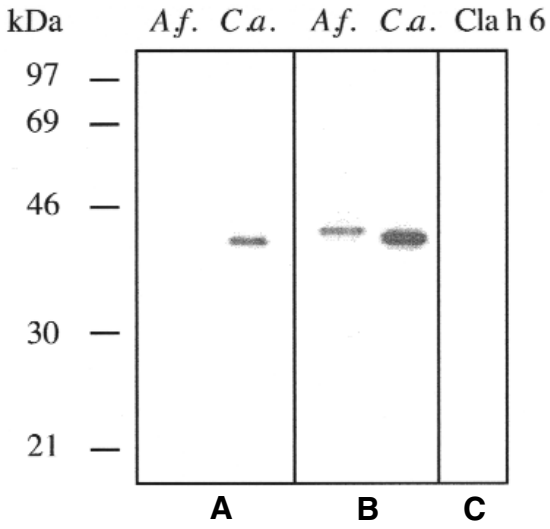

FIG 7. Cross-reactivity between *C herbarum* enolase and the enolases from *A fumigatus* and *C albicans*, respectively, was analyzed. **A,** rnfCla h 6–depleted serum was loaded onto membrane strips containing 10 μg of protein extract of *A fumigatus* and *C albicans*, respectively. **B,** Extracts of *A fumigatus* and *C albicans* were incubated with nondepleted patient's serum. **C,** rnfCla h 6–depleted serum was loaded onto a membrane strip containing 1 μg of rnfCla h 6.

lack of antibody binding. Looking at the modeled structure of *Cladosporium* enolase (Fig 4), it is shown that peptide 9 in the context of the enolase structure is spanning the whole diameter and reaching the surface at 2 opposite sites of the protein. Apparently, the rest of the sequence supports the correct folding of peptide 9. Somewhat unexpectedly, GST can also support the correct folding of the C-terminally fused peptide 9 as shown by its immunologic reactivity.

On the basis of the high sequence identity of 74% between the enolases of *C herbarum* (Cla h 6) and *S cerevisiae*, the 3-dimensional structure of Cla h 6 was modeled on the known structure of *S cerevisiae* enolase 1 (Fig 4). Analysis of the structure revealed that several areas of peptide 9 are exposed on the surface of the protein and could form a conformational epitope. Changing one of the exposed lysines to alanine and glutamine, respectively, in the context of the complete enolase sequence did not interfere with IgE binding, indicating that Lys141 is not in a critical position for the binding of IgE antibodies. Only an extensive investigation of parts of the 69 amino acid sequence discussed here with more in vitro mutagenized amino acids could lead to a more precise mapping of the epitope or epitopes.

Determination of the IgE reactivities of various fungal enolases by cross-inhibition experiments revealed that enolase is a highly cross-reactive fungal allergen (Figs 5-7). The cross-inhibition experiments show that the structure responsible for IgE binding is highly conserved between different fungal species. This is true for the one patient shown and for 3 more patients that showed exactly the same results. We therefore believe that the phenomenon of interspecies cross-reactivity of fungal enolases is a general one.

Competition between the 69-amino-acid fusion protein and the total *C herbarum* enolase showed that nearly all enolase-binding IgE antibodies were removed by prior binding to the fusion protein. In the same way IgE binding to *A alternata* enolase was also markedly inhibited. This shows that the major part of the IgE-binding epitopes of enolase is located in the short region defined here. We find it intriguing that the epitope or epitopes are dominant in the sense of binding most of the IgE antibodies directed against *C herbarum* enolase in a specific serum and are cross-reactive between different allergenic mold species. These results were obtained with one patient's serum and do not necessarily reflect the situation with anti-enolase IgE of the majority of patients.

On the basis of the amino acid sequence, it has never been possible to define why certain proteins become allergens and others that are also present in a given allergen source do not. The suspicion always has been that not the sequence per se but one or a relatively small number of allergenic 3-dimensional structures could be the criteria defining an allergen. The epitope or epitopes mapped here are dominant compared with the rest (about 90%) of the protein sequence and are even conserved between species and could be a first step toward identifying such an allergenic structure in molecular terms.

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