

Transaldolases are novel and immunoglobulin E cross-reacting fungal allergens

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Clinical & Experimental Allergy

Summary

Background Mould-induced atopic respiratory diseases are a worldwide problem. Characterization of fungal allergens is of major clinical importance.

Objective We identified a novel transaldolase family allergen of *Cladosporium* and *Penicillium* species.

Methods Fungal allergens were identified by immunoblotting, peptide mass mapping and partial sequencing, cDNA cloning and IgE epitope mapping.

Results A 36.5 kDa IgE-binding component in a partially purified *C. cladosporioides* preparation was identified. Mass spectrometric analyses suggest that this novel IgE-reacting allergen is a transaldolase. A corresponding full-length 1246 bp cDNA encoding a polypeptide of 325 residues was isolated. The newly identified transaldolase allergen has been designated as Cla c 14.0101. The cDNA encoding the *Penicillium chrysogenum* transaldolase was isolated by RT-PCR according to the cDNA sequence encoding a *P. chrysogenum* Wisconsin 54-1255 hypothetical protein. The purified rCla c 14.0101 protein reacted with IgE antibodies in 10 (38%) of 26 *Cladosporium cladosporioides*-sensitized asthmatic patients. Nine of the 10 rCla c 14.0101-positive sera have IgE binding against the recombinant *Penicillium* transaldolase (rPen ch 35.0101). Among the eight fungal transaldolase-positive sera tested, three showed IgE binding against the recombinant human transaldolase. To determine cross-reactivity between the *Cladosporium* and *Penicillium* fungi, IgE cross-reactivity was detected between these two fungal transaldolase allergens by inhibition assays. Both the N- and the C-terminal fragments of Cla c 14.0101 were recognized by IgE antibodies. The C-terminal IgE-reacting determinant was narrowed down to a region encompassing Thr257 to Ser278 of Cla c 14.0101. It was mapped onto a loop-like structure of a 3D model constructed for Cla c 14.0101.

Conclusion and Clinical Relevance We identified transaldolase as a novel and IgE cross-reactive allergen family of *C. cladosporioides* and *P. chrysogenum*. In addition, an IgE-reacting fragment (Thr257 to Ser278) was pinpointed to a loop-like structure on Cla c 14.0101. Results obtained provide important information in clinical mould allergy.

Keywords allergen, *C. cladosporioides*, *P. chrysogenum*, transaldolase

Submitted 25 July 2010; revised 21 December 2010; accepted 24 December 2010

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Cite this as: H. Chou, M. F. Tam, C.-H. Chiang, C.-T. Chou, H.-Y. Tai and H.-D. Shen, *Clinical & Experimental Allergy*, 2011 (41) 739–749.

Introduction

Fungi are ubiquitous in our environment. Atmospheric fungal spores existing in numbers greatly exceed those of environmental pollens have been identified as important causative agents of human respiratory atopic diseases [1, 2].

Sensitization to moulds has been reported in close to 80% of asthmatic patients [2, 3]. Characterization of fungal allergens plays a critical role in successful diagnosis and treatment of fungal allergy [4, 5].

Cladosporium and *Penicillium* species are common fungi in the air [2, 6, 7]. The group 13 alkaline serine proteases and the group 18 vacuolar serine proteases are major allergens of the prevalent *Penicillium* species [8, 9].

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Both groups of allergens can be recognized by IgE antibodies from >60% of *Penicillium*-allergic patients. The other important *Penicillium* allergens reported include the group 3 (peroxisomal membrane protein), group 19 (heat-shock protein P70), group 20 (*N*-acetylglucosaminidase), group 22 (enolase) and the group 24 (elongation factor 1- β) allergens [2, 8, 9].

In studies of *Cladosporium* allergens, an NADP-dependent mannitol dehydrogenase (Cla h 8) of *Cladosporium herbarum* is a major allergen that can be recognized by IgE antibodies from 57% of *Cladosporium*-allergic patients [2, 10]. Other minor allergens recognized by <20% of *C. herbarum*-allergic patients are ribosomal proteins P1 (Cla h 12) and P2 (Cla h 5), enolase (Cla h 6), flavodoxin (YCP4 homologue, Cla h 7), aldehyde dehydrogenase (Cla h 10), a cold-shock factor (Cla h CSP), glutathione-S-transferase (Cla h GST), type I hydrophobin (Cla h HCh1), heat-shock protein 70 (Cla h HSP70), nuclear transport factor 2 (Cla h NTF2), a vacuolar serine protease (Cla h 9) and a hydrolase [2, 11, 12].

Cladosporium cladosporioides sensitizes about 38% of atopic asthma patients [13]. Vacuolar serine protease (Cla c 9) is a major allergen of this prevalent airborne *Cladosporium* species [13]. Forty-one (55%) of 74 *Cladosporium*-allergic patients showed IgE binding against the Cla c 9 allergen [13]. An amino acid sequence identity of 97.6% was found between the vacuolar serine protease allergens of *C. cladosporioides* (Cla c 9) and *C. herbarum* (Cla h 9). In addition, IgE cross-reactivity was detected among Cla c 9 and serine protease allergens from *Aspergillus fumigatus* (Asp f 18) and *Penicillium chrysogenum* (Pen ch 18) [13]. Nonetheless, allergens of *C. cladosporioides* with molecular masses ranging from 16 to 88 kDa have been identified [14] and they have not been characterized.

In this study, a novel family of IgE-reacting protein from *C. cladosporioides* and *P. chrysogenum* was successfully identified. We present evidence that the novel *Cladosporium* and *Penicillium* allergens are transaldolases. In addition, we demonstrated the presence of IgE cross-reactivity between these conserved transaldolase fungal allergens.

Materials and methods

Serum samples

Serum samples used in the present study were collected from patients with bronchial asthma attending the allergy clinics of the Taipei Veterans General Hospital. Patients with bronchial asthma were diagnosed according to the statement provided by the American Thoracic Society [15]. Twenty-six serum samples that showed IgE immunoblot reactivity against *C. cladosporioides* components were stored in aliquots at -70°C and used in the present study. This study has been approved by the Institutional Review Board of the Taipei Veterans General Hospital.

Fungal extracts preparation and immunoblotting

C. cladosporioides strain BCRC 30812 (BCRC: the Biore-source Collection and Research Center, <http://www.ccrcc.firdi.org.tw/>) was isolated from the air of Taiwan and provided by the Food Industry Research and Development Institute, Hsinchu, Taiwan. It was cultured in a CYB medium containing yeast carbon base (Difco Laboratories, Detroit MI, USA; 11.7 g/L), casein enzymatic hydrolysate (Sigma Chemical Co., St Louis, MO, USA; 10 g/L) and glucose (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA; 10 g/L) without agitation at 26°C for 6 days. Crude and lyophilized *C. cladosporioides* extracts were prepared essentially as described previously [13]. *P. chrysogenum* (BCRC 30568) extracts were prepared similarly. The rDNA ITS1–5.8S–ITS2 sequences of both fungal species were determined. The sequences submitted to GenBank accession no. HQ 529707 for *C. cladosporioides* BCRC 30812 and HQ529708 for *P. chrysogenum* BCRC 30568 which showed 99.8% (546/547) and 100% (546/546) sequence identity to those of *C. cladosporioides* ATCC 66669 (AF393689) and *P. chrysogenum* NRRL 832 (AF034449), respectively, confirm the identity of the fungal cultures. The protein content of the extracts was determined with a Bradford dye-binding method (Coomassie brilliant blue G-250 dye) according to the manufacturer's instructions (Bio-Rad, Richmond, CA, USA).

Proteins in fungal extractions were separated by SDS-PAGE as described [13] then transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (0.45 μm , Millipore, Bedford, MS, USA). Immunoassay with human IgE antibodies against protein components was carried out using alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmingen, San Diego, CA, USA) as described previously [13]. The blots were developed in a substrate solution of carbonate buffer (0.1 M NaHCO_3 , 1.0 mM MgCl_2 , pH 9.8) containing 0.38 mM BCIP (5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt) and 0.41 mM NBT (*p*-nitro blue tetrazolium chloride) for about 30 min. For the detection of fungal transaldolase, the membranes were incubated with an affinity-purified goat polyclonal antibody against human transaldolase (T-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The blots were washed and incubated with peroxidase-conjugated mouse anti-goat IgG antibodies (Jackson ImmunoResearch, West Grove, PA, USA) and developed in a substrate solution of acetate buffer (50 mM, pH 5.0) containing 1 mM 3-amino-9-ethyl-carbazole (Sigma Chemical Co.) and 0.01% (v/v) hydrogen peroxide for about 5 min. For controls, serum of a non-atopic healthy individual and a normal goat serum were used.

Identification of novel allergens from fungal extracts

Chromatographic separation media were purchased from GE Healthcare (Uppsala, Sweden). Lyophilized crude *C.*

cladosporioides extracts were dissolved in Buffer A (20 mM Tris-HCl, pH 7.2, 0.5 M NaCl, 1 mM CaCl₂ and 1 mM MnSO₄) then loaded onto a Con A Sepharose 4B column equilibrated in Buffer A. The unbound fraction was dialysed against Buffer B (10 mM phosphate buffer, pH 6.3) before loading onto a DEAE Sepharose column pre-equilibrated in the same buffer. The unbound fraction from the DEAE Sepharose column was further separated on a denaturing 12.5% SDS-PAGE for immunoblot assay. A 36.5 kDa component reactive against IgE antibodies derived from *Cladosporium*-allergic patients was excised from the gel and subjected to trypsin digestion and mass spectrometric analyses [16] in an Autoflex III (Bruker Daltonic, Bremen, Germany) instrument with the matrix system described by Wu et al. [17]. Peptide masses obtained were searched against the NCBI non-redundant protein database with the MASCOT program for protein identification. Furthermore, ions from the peptide map were selected for MS/MS partial sequence analysis to affirm the database search results. The MS/MS spectra were manually inspected for fragment ions assignment.

Enzymatic activity of transaldolase

Transaldolase activity of the partially purified *C. cladosporioides* preparation (3.0 µg) was assayed in a 1.0 mL reaction mixture containing glycylglycine (250 mM, pH 7.7), D-fructose 6-phosphate (6.7 mM), D-erythrose 4-phosphate (2.0 mM), β-NADH (β-nicotinamide adenine dinucleotide, reduced form, 0.13 mM) and 3.3 µg of α-glycerophosphate dehydrogenase/triosephosphate isomerase. The reaction was carried out at room temperature and the decrease in A_{340 nm} was recorded for 5 min [18]. The measurement reflects the conversion of β-NADH to β-NAD and correlates with the transaldolase activity. As a negative control, bovine serum albumin (BSA, Pierce, Rockford, IL, USA) was used in place of the fungal preparation. All reagents for the transaldolase assay were obtained from Sigma Chemical Co.

Complementary deoxyribonucleic acid cloning

The cDNA encoding the *C. cladosporioides* transaldolase was isolated with an AffinityScript Multiple Temperature cDNA Synthesis kit (Stratagene, La Jolla, CA, USA) and PCR as described previously [13]. Primers used in the cloning experiments are listed in Table 1. Primers Cc-TAL-f and AP were used in the first set of PCR. The product obtained was used as a template in a subsequent PCR with primers Cc-TAL-f and AUAP. The product from the nest PCR reaction was gel purified and inserted into the pGEM-T vector (Promega, Madison, WI, USA) for sequencing analysis.

The full-length cDNA of the *C. cladosporioides* transaldolase was obtained by 5'-rapid amplification of cDNA end (RACE) reaction. The template cDNA for the reaction was

Table 1. Primers used in PCR

Name	Nucleotide sequence in 5'-3'-end orientation
Cc-TAL-f	aag/tac/aag/cc(a/c)/ca(a/g)/ga(t/c)/gc
AP	GGCCACGCGTCGACTAGTACT-(dT) ₁₆
AUAP	GGCCACGCGTCGACTAGTAC
TAL-GSP-r1	⁴⁷⁷ GGTGAAGCGAGCTTGATGAGAA ⁴⁵⁵
TAL-GSP-r2	³⁶² TCGACCTCAGTGGAGACCTT ³⁴³
AAP	GGCCACGCGTCGACTAGTAC GGGIIGGGIIGGGIIG
Cc-TAL-F1f	CGGGATCC ⁷⁰ tcttctctcctcgaccagctcaa ⁹²
Cc-TAL-F1r	AACTGCAG ⁴²⁰ tttgataatgcggaggccctt ⁴⁰⁰
Cc-TAL-F2f	CGGGATCC ³⁵² actgagtgacgcccgcctt ³⁷¹
Cc-TAL-F2r	AACTGCAG ⁷⁷¹ ttggatctcgcctcaatgtt ⁷⁵⁴
Cc-TAL-F3f	CGGGATCC ⁶⁸⁵ gtcgtcgacatcttcaac ⁷⁰²
Cc-TAL-F3r	AACTGCAG ¹⁰⁴¹ tttagcctctcgtatcttct ¹⁰²⁵
Cc-TAL-F3-1f	CGGGATCC ⁴²¹ ctctacaaggagcgccgcat ⁴⁴¹
Cc-TAL-F3-1r	AACTGCAG ⁸³⁴ ttgctgttagagctgctcaagaa ⁸¹²
Cc-TAL-F3-2r	AACTGCAG ⁹⁰⁰ ttgctcttctcgtatgctgag ⁸⁸⁰
Cc-TAL-F3-3r	AACTGCAG ⁹⁶⁶ ttgagctgttgacggccat ⁹⁴⁹
Pc-TAL-f	CGGGATCC ¹ tcttctctcctgaacagctta ²⁰
Pc-TAL-r	AACTGCAG ⁹⁷⁵ ctaacctggtatcttctgggcaag ⁹⁵²

synthesized with reverse transcriptase (RT, Stratagene) and primer TAL-GSP-r1. An oligo-(dC) was added to the end of the purified cDNA with terminal deoxynucleotidyl transferase (Promega). Primers AAP and TAL-GSP-r2 were then used in the 5'-RACE reaction. The product was purified, subcloned, transformed and subsequently sequenced.

The cDNA encoding the *P. chrysogenum* transaldolase was isolated by RT-PCR with total RNA as the template. The forward (Pc-TAL-f) and reverse (Pc-TAL-r) primers used in the reaction are listed in Table 1. The PCR product was purified, subcloned, transformed and sequenced as described above.

Preparation of recombinant fungal transaldolases

The transaldolases of *C. cladosporioides* (Cla c 14.0101) and *P. chrysogenum* (Pen ch 35.0101) were expressed with an N-terminal His₆-tag. The forward and the reverse primers used in the PCR amplification of Cla c 14.0101 (Cc-TAL-F1f and Cc-TAL-F3r, respectively) and Pen ch 35.0101 (Pc-TAL-f and Pc-TAL-r, respectively) are listed in Table 1. The PCR products were restricted then ligated into the pQE-80 vector (Qiagen Inc., Valencia, CA, USA) for protein expression in *Escherichia coli* JM109 cells. The recombinant Cla c 14.0101 and Pen ch 35.0101 were affinity-purified with Ni-NTA resin columns (Qiagen) according to the manufacturer's instructions. Immunoreactivity of the fungal transaldolase recombinant proteins [rCla c 14.0101 (0.4 µg/strip), rPen ch 35.0101 (0.2 µg/strip)] and the human transaldolase recombinant protein [Novus Biologicals, Littleton, CO, USA (0.5 µg/strip)] against IgE antibodies and goat polyclonal antibody against human transaldolase (Santa Cruz Biotechnology) was analysed by SDS-PAGE-immunoblot.

Immunoblot inhibition

For immunoblot inhibition studies, IgE-containing serum samples were firstly incubated with purified rCla c 14.0101 or rPen ch 35.0101 before incubating with PVDF blots containing partially purified *C. cladosporioides* or *P. chrysogenum* extracts or purified rCla c 14.0101 at 4 °C for 16 h. As controls, the blots were incubated with similar serum samples that have been pre-incubated with equivalent amounts of BSA (Pierce). The blots were then washed and incubated with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmingen, San Diego, CA, USA) and developed with enzyme substrates as described previously [13].

Immunoglobulin E epitope mapping

Recombinant proteins covering three overlapping fragments of Cla c 14.0101 were prepared to map the antigenic fragment(s) recognized by human IgE antibodies. Fragments encompassing amino acid residues 2–118 (F1), 96–235 (F2) and 207–325 (F3) of Cla c 14.0101 were expressed as His₆-tagged proteins and purified essentially as described above. The cDNA inserts of these fragments were generated with PCR using three separate sets of primers (Table 1). These primers are Cc-TAL-F1f and Cc-TAL-F1r, Cc-TAL-F2f and Cc-TAL-F2r and Cc-TAL-F3f and Cc-TAL-F3r, for fragments F1, F2 and F3, respectively. The PCR products were purified and restricted with *Bam*HI and *Pst*I. They were ligated into the pQE80 vector (Qiagen) and transformed into *E. coli* JM109 for protein expression. Antigenic fragment(s) within F3 were further determined with recombinant proteins covering four overlapping fragments of Cla c 14.0101. The cDNA inserts of these fragments were generated with PCR using four separate sets of primers (Table 1). The forward primer used is Cc-TAL-F3-1f. The reverse primers used are Cc-TAL-F3-1r, Cc-TAL-F3-2r, Cc-TAL-F3-3r and Cc-TAL-F3r for fragments F3-1 (amino acid residues 119–256), F3-2 (amino acid residues 119–278), F3-3 (amino acid residues 119–300) and F3-4 (amino acid residues 119–325), respectively. The recombinant proteins of F3-1, F3-2, F3-3 and F3-4 were prepared and purified as described above. In addition to the Cla c 14.0101-encoded amino acid residues, all recombinant proteins of various Cla c 14.0101 fragments also contained an additional vector-derived N-terminal tag of RGSHHHHHGS. Recombinant proteins of Cla c 14.0101 (0.4 µg/strip) and various Cla c 14.0101 fragments (0.2 µg/strip) recognized by human IgE antibodies were analysed by SDS-PAGE immunoblotting as described above. In addition, a peptide covering the amino acid sequence from Thr257 to Ser278 (TDAVPQKLKAED-VAKLDIEKKS) of Cla c 14.0101 was also custom synthesized (Genemed Synthesis Inc., San Antonio, TX, USA) and used in the mapping of IgE epitopes by immunodot blotting. Briefly,

50 µg of the peptide and 5 µg of rCla c 14.0101 were dotted, individually, onto nitrocellulose membranes (0.2 µm, Bio-Rad) using a Bio-Dot microfiltration apparatus (Bio-Rad) according to the manufacturer's instructions. The membranes were blocked with 2% skimmed milk. Immunoassay with human IgE antibodies against dotted peptides and proteins was carried out as described above in SDS-PAGE immunoblot assay. The control peptide (50 µg/dot) used contains the amino acid sequence ³¹PMKVPDHSKFERHI⁴⁵ of the house dust mite allergen Der p 7.

Homology-based modelling of antigenic fragments recognized by immunoglobulin E antibodies

Molecular modelling of Cla c 14.0101 was performed using Swiss-Model programs in the first approach mode [19, 20]. The coordinates of a transaldolase (3cq0A) from *Saccharomyces cerevisiae* with 65% amino acid sequence identity with Cla c 14.0101 (analysed by the CLUSTAL-W program) were used in the computation. The model obtained was verified by PROCHECK [21] using Ramachandran plots [22]. The smallest antigenic fragment recognized by IgE antibodies was visualized and displayed with the WebLab Viewer version 3.1 (Accelrys, San Diego, CA, USA) or VMD molecular visualization program (VMD1.8.6) (<http://www.ks.uiu.edu/Research/vmd/>) [23].

Results

Identification of a novel transaldolase allergen from *Cladosporium cladosporioides*

Crude *C. cladosporioides* extracts were fractionated through Con A Sepharose 4B, then DEAE Sepharose columns. The unbound fraction from the DEAE Sepharose columns was separated by SDS-PAGE and transferred onto PVDF membranes for immunoblotting experiments. Results in Fig. 1 demonstrate that a 36.5 kDa *C. cladosporioides* component reacted with human IgE antibodies in a pooled serum sample of *C. cladosporioides*-sensitized asthmatic patients (Fig. 1). For control, serum from a non-atopic healthy individual showed negative IgE immunoblot reactivity (data not shown). In addition, this 36.5 kDa component did not react with monoclonal antibody FUM20, which reacts with the 36 kDa major vacuolar serine protease allergen (Cla c 9) of *C. cladosporioides* [13] (data not shown).

This 36.5 kDa IgE-reacting component was subjected to trypsin in-gel digestion and mass spectrometric analyses. The mass spectrum of the resulting peptides is shown in Fig. 2. Ions with mass (837.4, 876.4, 935.5, 981.5, 997.5 and 1918.0) that match with those derived from *A. fumigatus* transaldolase are marked (#). Ten ions, including five out of the six mentioned above were selected for MS/MS partial sequencing analysis. A minimum of five consecutive residues can be identified by the fragment

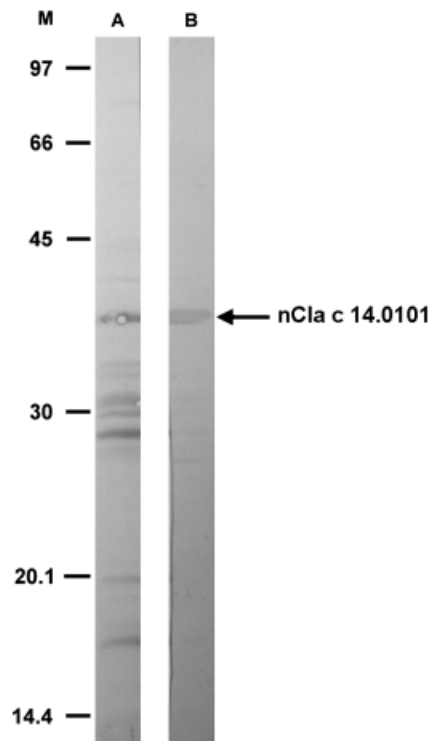


Fig. 1. Immunoblot reactivity of IgE antibodies in a pooled serum sample obtained from six asthmatic patients (B) against component(s) in a partially purified *Cladosporium cladosporioides* extracts. (A) Dye-stained protein profile of the *C. cladosporioides* preparation on a polyvinylidene difluoride (PVDF) membrane. In addition to the 36.5 kDa IgE-binding protein, the partially purified preparation contains other *C. cladosporioides* protein components.

ions from each parental ion. The results are shown in Table 2 and suggest that the 36.5 kDa IgE-binding component is a transaldolase of *C. cladosporioides*.

Transaldolase activity of the partially purified *Cladosporium cladosporioides* preparation

In the transaldolase assay, a decrease in absorbance at 340 nm reflects the activity of the enzyme. The $A_{340\text{ nm}}$ for the partially purified *C. cladosporioides* preparation, the BSA and the reaction buffer at the beginning of the reaction were 0.75, 0.79 and 0.76, respectively. After 5 min of incubation, the $A_{340\text{ nm}}$ for the partially purified preparation, BSA and the buffer control were 0.18, 0.72 and 0.70, respectively. Results obtained indicate the presence of transaldolase (EC 2.2.1.2) in the partially purified preparation of *C. cladosporioides*.

Complementary deoxyribonucleic acid cloning

The 1246 bp cDNA encoding the *C. cladosporioides* transaldolase was constructed by RT-PCR coupled with the 5'-end RACE reaction. The nucleotide (GenBank accession no. GQ906475) and the deduced amino acid sequence of the open reading frame (from bases 67 to 1041) are shown in Fig. 3. A potential polyadenylation signal (AATCAA) for mRNAs from higher eukaryotes is located 14–19 bases upstream from the poly-A tail. Without the initiator methionine [24], the mature protein starting from Ser2 has 324 residues and a calculated molecular mass of

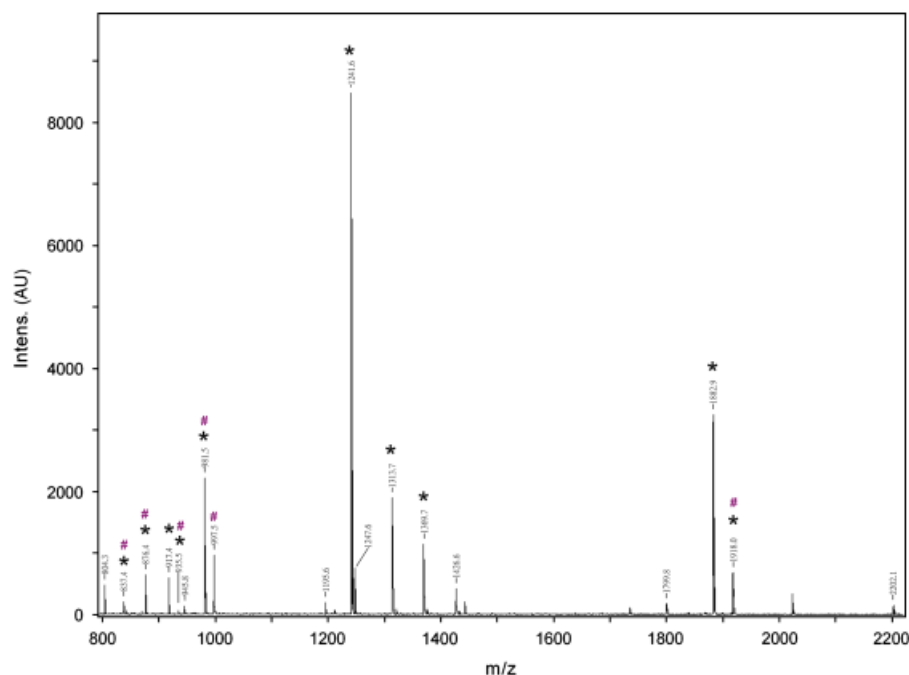


Table 2. Summary of mass spectrometric analyses of tryptic digests from partially purified *Cladosporium cladosporioides* transaldolase

Sequence	m/z	
	Observed	Calculated
¹⁸⁴ ILDWYK ^{189*} †	837.4	837.5
⁹⁴ VSTEVDAR ^{101*} †	876.4	876.4
¹²² EAGIDKER ^{129*} †	917.4	917.5
³⁰⁷ FAADAVTLK ^{315*} †	935.5	935.5
²²¹ TIVMGASFR ^{229*} †	981.5	981.5
²²¹ TIVM(O)GASFR ^{229†}	997.5	997.5
²⁷⁸ SYLKDEALFR ^{287*} †	1241.6	1241.6
²¹⁷ YDYKTIVMGAS ²²⁷	1247.6	1247.6
¹⁰² FSFDKDVSIQK ^{112*} †	1313.7	1313.7
²⁷⁷ KSYLKDEALFR ^{287*} †	1369.7	1369.7
^{134†} LASTWEGIQAAHELQTK ^{150*}	1882.9	1883.0
²⁸ YKPDATTNPSLILAASK ^{45*} †	1918.0	1918.0

*Peptides subjected to MS/MS partial sequencing analysis.

†Peptides with mass match to that of *Aspergillus fumigatus* transaldolase.

35 927 Da, excluding further post-translational modifications. It has three cysteines (Cys17, Cys155 and Cys240) and three putative *N*-glycosylation sites (¹⁵⁶NLT¹⁵⁸, ¹⁹⁷NAS¹⁹⁹ and ²⁵⁵NST²⁵⁷) (Fig. 3). In addition, conserved residues among transaldolases (Asp18, Asn36, Glu97, Lys133, Asn156, Thr158, Ser178 and Arg183) that are crucial to catalysis or substrate binding [25] can also be readily located (Fig. 3). It has been designated as Cla c 14.0101 according to the rules of the I.U.I.S. Allergen Nomenclature Sub-committee.

In this study, the full-length cDNA of the transaldolase from *P. chrysogenum* was also isolated by RT-PCR (data not shown). The nucleotide sequence of the isolated cDNA (accession no. GQ925430) is identical to that of a hypothetical protein of *P. chrysogenum* (accession no. XM_002568649). The putative mature protein without the initiator methionine has 323 residues and a calculated molecular mass of 35 309 Da, excluding further post-translational modifications. The *P. chrysogenum* transaldolase has been named as Pen ch 35.0101 by the I.U.I.S. Allergen Nomenclature Sub-committee.

Sequence alignment revealed that the *C. cladosporioides* transaldolase has 210 (65%), 244 (75%), 248 (76%) and 199 (61%) amino acids identical to that of *S. cerevisiae* (accession no. AAB67752), *A. fumigatus* (accession no. XM_748 623), *P. chrysogenum* (accession no. GQ925430) and *Homo sapiens* (accession no. AAF40478) transaldolase, respec-

tively (data not shown). The three cysteine residues are conserved among *A. fumigatus*, *P. chrysogenum* and *C. cladosporioides* transaldolases. However, the transaldolases from *S. cerevisiae* and *Homo sapiens* contain only one and two cysteine residues, respectively (data not shown). The three potential *N*-glycosylation sites of *C. cladosporioides* transaldolase are boxed in Fig. 3. Only the first putative glycosylation site (¹⁵⁶NLT¹⁵⁸) is conserved among these enzymes. Because the *C. cladosporioides* transaldolase was recovered in the unbound fraction of the Con A column, this protein is most likely not a glycosylated protein.

Immunoreactivity and transaldolase enzymatic activity of rCla c 14.0101 and rPen ch 35.0101

Both purified recombinant transaldolases rCla c 14.0101 and rPen ch 35.0101 (Fig. 4A) reacted with a goat polyclonal antibody against human transaldolase (Fig. 4B, strip 1). For control, a normal goat serum showed negative immunoblot reactivity against both recombinant proteins (Fig. 4B, strip 2). The purified rCla c 14.0101 protein also reacted with IgE antibodies in the sera of 10 (38%) out of 26 *C. cladosporioides*-sensitized asthmatic patients (Fig. 4C). Furthermore, sera from nine (35%) of the same 26 *P. chrysogenum*-sensitized patients have IgE binding against the rPen ch 35.0101 (Fig. 4). For control, serum from a non-atopic healthy individual showed negative IgE immunoblot reactivity against rCla c 14.0101 and rPen ch 35.0101 (Fig. 4C, strip no. 11). Furthermore, results in Fig. 4 also showed that three (Fig. 4C, strip no. 4, 5 and 9) of the eight patients' sera tested also demonstrated IgE reactivity against the human transaldolase recombinant protein, which reacted with a goat polyclonal antibody against human transaldolase (Fig. 4B, strip 1).

In the transaldolase enzymatic activity assay, the $A_{340\text{ nm}}$ for the purified rCla c 14.0101 and rPen ch 35.0101, the BSA and the reaction buffer at the beginning of the reaction were 0.47, 0.48, 0.47 and 0.47, respectively. After 5 min of incubation, the $A_{340\text{ nm}}$ for rCla c 14.0101, rPen ch 35.0101, BSA and the buffer control were 0.20, 0.20, 0.44 and 0.44, respectively. Results obtained indicate the presence of transaldolase activity in the purified rCla c 14.0101 and rPen ch 35.0101.

Immunoglobulin E cross-reactivity

Results in Fig. 5 show that both nCla c 14.0101 and nPen ch 35.0101 in partially purified fungal extracts reacted with

Fig. 3. Nucleotide and deduced amino acid sequences of Cla c 14.0101 (GenBank accession no. GQ 906475). Numbers to the right indicate the positions of the nucleotides and the deduced amino acid residues of the sequences. Amino acid residues that are conserved and involved in transaldolase catalysis and substrate binding are depicted in bold letters and shaded. Potential *N*-glycosylation sites are in bold letters and boxed. Amino acid residues that can be inferred from the peptide map as shown in Fig. 2 and summarized in Table 2 are underlined. Amino acid residues corresponding to those of *Aspergillus fumigatus* transaldolase inferred from mass spectrometric analysis as shown in Table 2 are in *italics*. The stop codon TAA is denoted with an *. Nucleotides in grey correspond to those synthesized and used as primers for PCR in the cDNA cloning of Cla c 14.0101 as shown in Table 1. The sequences corresponding to primers Cc-TAL-F1f and Cc-TAL-F3r used in the preparation of rCla c 14.0101 are boxed.

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gacgatttatcggtaccttcttctcttgcattaccctcacaacctctctcatacacacca 60
gcaatc 66
atgtcttctcctccctcgaccagctcaaggcctccggcaccaccgtcgtctgcgactccggt 126
M S S S L D Q L K A S G T T V V C D S G 20
gacttcgccaccattggcaagtacaagcctcaggatgccaccaccaacccctccctcattc 186
D F A T I G K Y K P Q D A T T N P S L I 40
ctcgccgcctccaagaagcccgagtacgccaagctcattgacgatgctgtcgccctacggt 246
L A A S K K P E Y A K L I D D A V A Y G 60
atgaagcacggcaagaccaccgaggagaagatcgatgccaccttcgacaacctcctcggt 306
M K H G K T T E E K I D A T F D N L L V 80
cagttcggtagcgagatcctcaagatcgtecccggaagggtctccactgaggtcgacgcc 366
Q F G S E I L K I V P G K V S T E V D A 100
cgcttctccttcgacaaggatgtctccatccagaaggccctccgcattatcgacctctac 426
R F S F D K D V S I Q K A L R I I D L Y 120
aaggaggccggcattgacaaggagcggttctctcatcaagctcgcttccacctgggagggt 486
K E A G I D K E R V L I K L A S T W E G 140
atccaggccgctcacgagctccagaccaagcacggcatcaactgcaacttgacctcatg 546
I Q A A H E L Q T K H G I N C N L T L M 160
ttctcctccccaggccattgcccgcgcgaggccggtgcctacctcatctctcccttc 606
F S L P Q A I A A A E A G A Y L I S P F 180
gtcgccgctatcctcgactggtacaaggccaacaccaagcaggagtacaacgcctccaac 666
V G R I L D W Y K A N T K Q E Y N A S N 200
gaccccggtgtcaagtccgtcgtcgacatcttcaactactacaagaagtagcactacaag 726
D P G V K S V V D I F N Y Y K K Y D Y K 220
accattgtcatggcgccctccttccgcaacattggcgagatcaccgagctcgccggctgc 786
T I V M G A S F R N I G E I T E L A G C 240
gactacctcaccatcgccccctgcccttcttgagcagctctacaacagcaccgatgccgttc 846
D Y L T I A P A L L E Q L Y N S T D A V 260
cccagaagctcaaggccgaggacgtcgccaagctcgacatcgagaagaagagctacctc 906
P Q K L K A E D V A K L D I E K K S Y L 280
aaggacgaggccctcttccgcttcgacttcaacgaggaccccatggccgtcaacaagctc 966
K D E A L F R F D F N E D P M A V N K L 300
tcgagggtatcagcaagtctcgtgccgacgccgtcacctcaaggacatcctccgagag 1026
S E G I S K F A A D A V T L K D I L R E 320
aagatcgagaaggcttaa 1044
K I E K A * 325
atgcctagtctcgtcgacgcttcttctcatggatttttaagaaagattgtcctacatttta 1104
cgtgcaatgatggtggatgacgagacagaatgcatacggtctcccaaacgagtagtcta 1164
tacatgtcttttttcttgaactattataccaatcaaattgggaattgtcaaaaaaaaa 1224
aaaaaaaaaaaaaaaaaaaaaaaaa 1246

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the goat polyclonal antibody against human transaldolase [Fig. 5, strips B of (I)] and human IgE antibodies [Fig. 5, strips C of (I)]. The rCla c 14.0101 inhibited, dose depen-

dently, IgE binding against nCla c 14.0101 when a pooled positive serum sample of Fig. 4 (nos. 2, 3, 4, 7, 9 and 10) was pre-absorbed with 2 and 10 µg of rCla c 14.0101 (Fig.

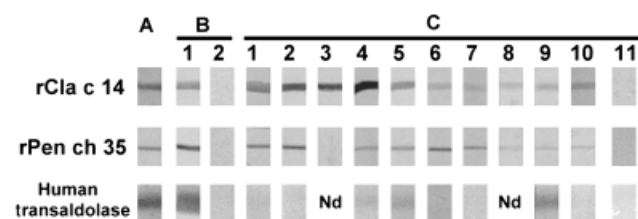


Fig. 4. Immunoblot reactivity of polyclonal goat anti-transaldolase (B) and IgE antibodies (C) in 10 serum samples from *Cladosporium clado-sporioides* and *Pencillium chrysogenum*-sensitized asthmatic patients against rCla c 14.0101, rPen ch 35.0101 and a recombinant human transaldolase protein. Strips of (A) show the dye-stained protein profiles of rCla c 14.0101 (0.4 µg/strip), rPen ch 35.0101 (0.2 µg/strip) and a recombinant human transaldolase protein (0.5 µg/strip) on polyvinylidene difluoride (PVDF) membranes. For control, a normal goat serum (B, strip 2) and serum from a non-atopic healthy individual (C, strip 11) were used in immunoblotting. Nd, not done.

5I). Pre-absorption of the same serum pool with 10 µg of BSA did not inhibit its IgE binding against nCla c 14.0101 (Fig. 5I). Similarly, the rPen ch 35.0101 inhibited, dose dependently, IgE binding against the nPen ch 35.0101 protein when serum no. 10 of Fig. 4 was pre-absorbed with 2 and 10 µg of rPen ch 35.0101 (Fig. 5I). Pre-absorption of the same serum sample with 10 µg of BSA did not inhibit its IgE binding against nPen ch 35.0101 (Fig. 5I).

IgE cross-reactivity was detected between *C. clado-sporioides* and *P. chrysogenum* transaldolases. The results shown in (II) of Fig. 5 demonstrated that pre-absorption of a mixture of serum samples containing IgE antibodies against rCla c 14.0101 (serum nos. 1, 2, 7, 8 and 10 in Fig. 4) with either rCla c 14.0101 (2 and 20 µg) or rPen ch 35.0101 (2 and 20 µg) inhibited, dose dependently, IgE binding against rCla c 14.0101. Inhibition of IgE binding was not detected when the same serum was pre-absorbed with 20 µg of BSA (Fig. 5II).

Immunoglobulin E epitope mapping

Cla c 14.0101 fragments recognized by human IgE antibodies were determined. Our results demonstrate that three (serum nos. 1, 3 and 6) and two (serum nos. 4 and 5) sera from six asthmatic patients with IgE reactivity against Cla c 14.0101 showed IgE binding to the F1 and the F3 fragments of Cla c 14.0101, respectively (Fig. 6I and II). In addition, four overlapping fragments (Fig. 6I) were constructed to map the linear epitope(s) within the F3 fragment of Cla c 14.0101. Our results demonstrate that only serum no. 5 has IgE reactivity against the F3-2, F3-3 and F3-4 fragments of Cla c 14.0101 (Fig. 6II). It indicates that IgE antibodies reacted, at least, with the Thr257 to Ser278 region of Cla c 14.0101. This finding was confirmed by results shown in Fig. 6 that serum no. 5 showed positive and serum no. 4 showed negative IgE immunodot blot reactivity against the peptide Thr257–Ser278 (50 µg/

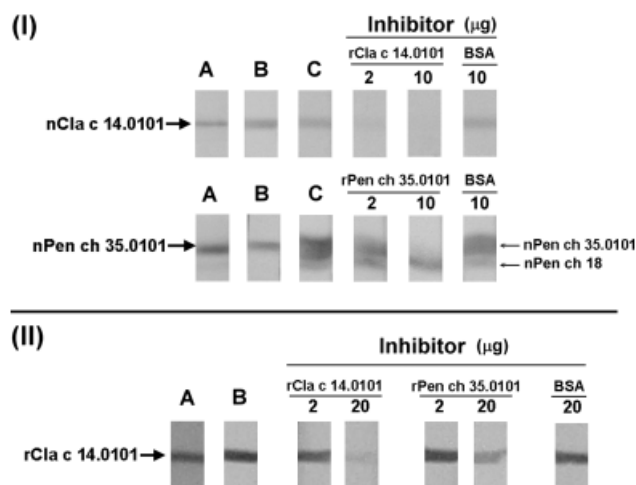


Fig. 5. Inhibition of IgE-immunoblot reactivity against nCla c 14.0101 and nPen ch 35.0101 with purified rCla c 14.0101 and rPen ch 35.0101 (2 and 10 µg) as inhibitors, respectively (I). IgE binding against nCla c 14.0101 and nPen ch 35.0101 using a pooled serum sample and serum no. 10 of Fig. 4, respectively, is shown in strips C of (I). Inhibition of IgE-immunoblot reactivity of a pooled serum sample against rCla c 14.0101 (strip B) by using rCla c 14.0101, rPen ch 35.0101 (2 and 20 µg) and bovine serum albumin (BSA, 20 µg) as inhibitors was shown in (II). Strips of panel A show the dye-stained protein profiles of nCla c 14.0101, nPen ch 35.0101 and rCla c 14.0101 on polyvinylidene difluoride (PVDF) membranes. Strips B of (I) show the immunoblot reactivity of polyclonal goat anti-transaldolase against nCla c 14.0101 and nPen ch 35.0101.

dot). For control, serum (no. 7) from a non-atopic healthy individual showed negative IgE reactivity against the full-length rCla c 14.0101, the fragments F1, F3, F3-1, F3-2, F3-3, F3-4, the Cla c 14.0101 peptide Thr257–Ser278 and the control Der p 7 peptide Pro31–Ile45 (Fig. 6II).

In this study, a homology-based model of the tertiary structure of the *Cladosporium* transaldolase allergen was generated to display the location of the IgE-reacting epitopes. The IgE-binding fragment encompassing Thr257 to Ser 278 is displayed on a 3D model of Cla c 14.0101 (Fig. 6III) constructed with a *Saccharomyces cerevisiae* transaldolase (3cq0A) as template and verified by PROCHECK [21]. In the Ramachandran plot made with PROCHECK, 92.5%, 7.2%, 0.3% and 0.0% of the residues were in the most favoured, additional allowed, generously allowed and disallowed regions, respectively, which confirms the accuracy of the modelled structure. The model of Cla c 14.0101 has an eight-stranded α/β barrel similar to that of other class I aldolases [24, 25]. The IgE-reacting fragment Thr257–Ser278 locates on a loop-like structure of the Cla c 14.0101 model (Fig. 6III).

Discussion

The present study identified and characterized a novel transaldolase family of allergens from prevalent *Cladosporium* and *Penicillium* species. About 38% of *C.*

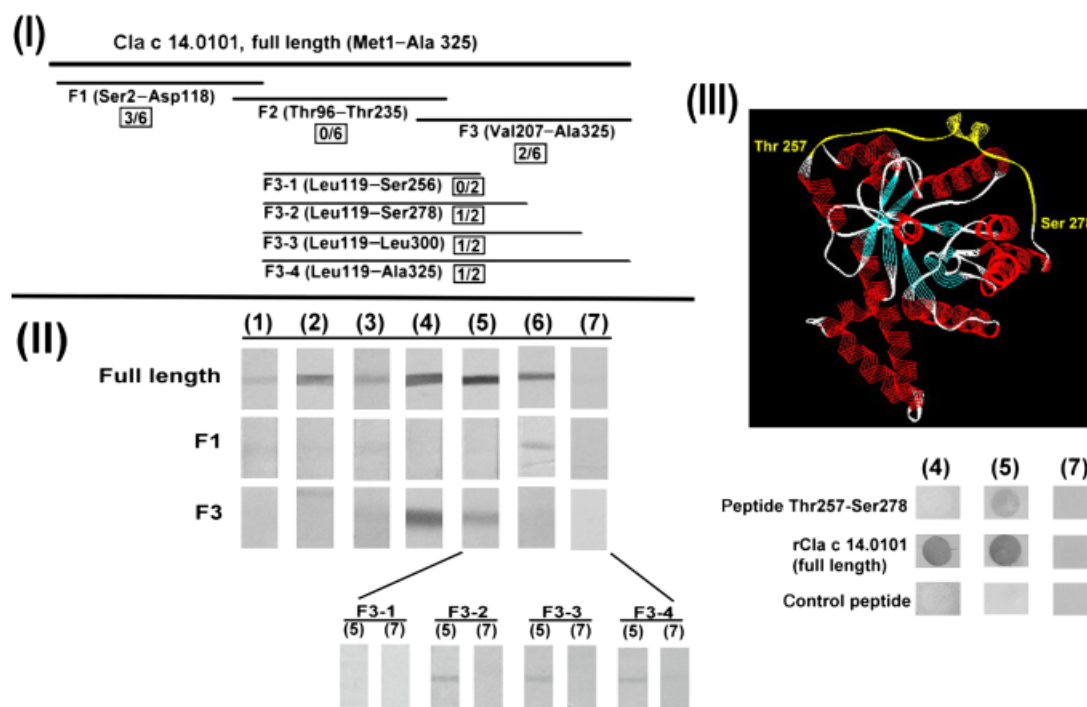


Fig. 6. SDS-PAGE-immunoblot reactivity of IgE antibodies against different fragments with overlapping sequences of the Cla c 14.0101 allergen. The numbers in the boxes in (I) show the frequency of IgE recognition. IgE binding against the full-length (0.4 µg/strip) and truncated fragments of rCla c 14.0101 (0.2 µg/strip) analysed by using six sera containing IgE antibodies against rCla c 14.0101 is shown in (II). IgE reactivity against the Cla c 14.0101 peptide Thr257-Ser278 and the control peptide (50 µg/dot) was determined by immunodot blotting. For control, serum from a non-atopic healthy individual was used (b, strip 7). A ribbon diagram of a structural model of the Cla c 14.0101 allergen is shown in (III). The region corresponding to an IgE epitope is coloured in yellow.

cladosporioides-sensitized asthmatic patients showed IgE binding against the transaldolase allergen. It indicates that besides Cla c 9 [13], the major vacuolar serine protease allergen, transaldolase is also an important allergen of *C. cladosporioides*.

Transaldolase (EC 2.2.1.2) catalyses the reversible transfer of a dihydroxyacetone moiety from a ketose donor to an aldose acceptor. Together with transketolase, it provides a link between the glycolytic and the pentose-phosphate pathways. Transaldolase is an enzyme of about 36 kDa with a well-conserved sequence throughout evolution. We cloned the full-length cDNAs encoding the transaldolases from *C. cladosporioides* and *P. chrysogenum*. The encoded transaldolases have calculated molecular weights close to that observed for the IgE-reacting component on the SDS-PAGE gel. In addition, the recombinant transaldolase (rCla c 14.0101) inhibited dose dependently IgE binding against the 36.5 kDa component of *C. cladosporioides*. Furthermore, transaldolase activity was detected in the partially purified *C. cladosporioides* preparation containing the 36.5 kDa IgE-reacting protein and the recombinant Cla c 14.0101 proteins. These observations support the notion that the 36.5 kDa allergen is a transaldolase of *C. cladosporioides* and much of the antigenicity of nCla c 14.0101 is retained by rCla c 14.0101.

The full-length cDNA encoding the *P. chrysogenum* transaldolase was also isolated by RT-PCR. Our results obtained from the present study also suggest that the 36.5 kDa IgE-reacting transaldolase is an important allergen of *P. chrysogenum*, which showed IgE cross-reactivity with Cla c 14.0101. This *P. chrysogenum* transaldolase has been reported previously as a hypothetical protein (accession no. CAP96592). A similar observation of IgE cross-reactivity has been reported for alkaline/vacuolar serine protease major allergens among *Penicillium*, *Aspergillus*, *Rhodotorula* and *Cladosporium* species [8, 9]. It provides an explanation for the clinical observation that mould-allergic patients often demonstrate sensitization to more than one fungal species [26, 27].

Dissecting of IgE-reacting determinants may help in understanding the allergenicity of allergens and in designing hypoallergenic molecules in immunotherapy of atopic allergy. Results obtained in the present study indicate the presence of a linear IgE-reacting fragment (Thr257-Ser278) on Cla c 14.0101. This IgE-reacting determinant was further mapped onto a loop-like structure of a Cla c 14.0101 structural model (Fig. 6). This finding correlates with the importance of loop-like structure on IgE binding and the allergenicity of allergens reported [28–30]. Interestingly, the most prominent

epitope (residues 271–285) of human transaldolase recognized by autoantibodies from multiple sclerosis patients is located on the surface of the enzyme [31] and corresponds to residues Pro261 to Glu275 of Cla c 14.0101.

In the present study, three patients with specific IgE to the fungal transaldolase were also sensitized to human transaldolase (Fig. 4). It was found that certain environmental allergens, including profilin, manganese superoxide dismutase and ribosomal P2 protein, were also identified as evolutionarily conserved IgE-reactive human antigens [32, 33]. IgE recognition of these corresponding human proteins might play a role in the stimulation and augmentation of type I hypersensitivity reactions without exogenous allergen exposure [33]. It might also contribute to the chronicity of certain severe allergic disorders [32]. However, further studies are needed to clarify in detail the presence and the significance of autoantigenic effects from these fungal allergens.

In conclusion, transaldolases have been identified as novel and important allergens of the prevalent *Cladosporium* and *Penicillium* species. IgE cross-reactivity between these highly conserved transaldolase allergens was detected. Furthermore, an IgE-reacting fragment on a loop-like structure of Cla c 14.0101 was characterized. Results obtained certainly provide important and valuable information in better diagnosis and improving immunotherapeutic strategies in clinical fungal allergy.

Acknowledgements

This work was supported by grants from the National Science Council (Grant NSC95-2320-B-075-005-MY3) and the Taipei Veterans General Hospital (V99C1-018), Taipei, Taiwan.

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