

Cross-Reactivity and 1.4-Å Crystal Structure of *Malassezia sympodialis* Thioredoxin (Mala s 13), a Member of a New Pan-Allergen Family¹

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We have identified thioredoxins (Trx) of *Malassezia sympodialis*, a yeast involved in the pathogenesis of atopic eczema, and of *Aspergillus fumigatus*, a fungus involved in pulmonary complications, as novel IgE-binding proteins. We show that these Trx, including the human enzyme, represent cross-reactive structures recognized by serum IgE from individuals sensitized to *M. sympodialis* Trx. Moreover, all three proteins were able to elicit immediate-type allergic skin reactions in sensitized individuals, indicating a humoral immune response based on molecular mimicry. To analyze structural elements involved in these reactions, the three-dimensional structure of *M. sympodialis* Trx (Mala s 13) has been determined at 1.4-Å resolution by x-ray diffraction analysis. The structure was solved by molecular replacement and refined to a crystallographic *R* factor of 14.0% and a free *R* factor of 16.8% and shows the typical Trx fold. Mala s 13 shares 45% sequence identity with human Trx and superposition of the solved Mala s 13 structure with those of human Trx reveals a high similarity with a root mean square deviation of 1.11 Å for all Cα atoms. In a detailed analysis of the molecular surface in combination with sequence alignment, we identified conserved solvent-exposed amino acids scattered over the surface in both structures which cluster to patches, thus forming putative conformational B cell epitopes potentially involved in IgE-mediated cross- and autoreactivity. *The Journal of Immunology*, 2007, 178: 389–396.

Thioredoxins (Trx)⁴ are small redox proteins found in all living cells. They undergo NADPH-dependent reduction by Trx reductase and in turn reduce oxidized cysteine groups on target proteins. The catalytic activity of Trx is based on the two redox-active cysteines in the highly conserved sequence WCGPC. A nucleophilic attack by the thiolate of the first cysteine breaks the disulfide bridge of the target protein, forming a mixed disulfide intermediate. This intermediate is then broken by the second cysteine, leaving the target protein reduced and releasing Trx in the oxidized form (1, 2). Activity has been found outside the cell (cell growth stimulation and chemotaxis), in the cytoplasm (as an antioxidant), in the nucleus (regulation of transcription factor activity), and in the mitochondria (3).

Trx has been identified as a new pan-allergen family able to elicit IgE-mediated hypersensitivity reactions from several species. Trx was first reported as an allergenic protein of *Coprinus comatus* (4). Trx from the mold *Aspergillus fumigatus*, the etiologic agent identified in the majority of *Aspergillus*-related human diseases (5), and from *Malassezia sympodialis*, a skin-colonizing yeast involved in the pathophysiology of atopic eczema (AE) (6), were isolated from fungal cDNA libraries displayed on phage surface (7). However, the allergenicity of these proteins has not been further analyzed. Screening of wheat germ and maize endosperm phage surface-displayed cDNA libraries with IgE from allergic bakers revealed that wheat and maize Trx are allergenic molecules able to cross-react with the homologous human enzyme (8) which represent, so far, the only available report on Trx allergenicity. Cross-reactivity between allergens and closely related homologous human Ags is often observed in allergic individuals suffering from chronic atopic diseases (8–11) and potentially contribute to exacerbation and/or perpetuation of chronic allergic reactions (6, 10). These studies provide strong evidence for in vitro and in vivo humoral and cell-mediated IgE autoreactivity in patients suffering from long-lasting atopic diseases (8, 11). Structural studies have shown that the availability of the crystal structure of a given allergen and its human homolog allows a detailed definition of the residues potentially involved in cross-reactivity (12). To date, the atomic details of the interaction between Ab and Ag are known for >30 Ab-Ag complexes (13). The B cell epitopes are, in all cases described, conformational and made up of residues that lie on different surface loops forming discontinuous B cell epitopes. They occupy a buried surface of 560–860 Å² and consist of 10–20 aa that are in contact with Ab residues (14). The only method to determine the complete structure of a B cell epitope is to cocrystallize the allergen with mAb Fab and solve the x-ray structure of the complex. The first structure of an allergen-Fab complex solved

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Received for publication March 29, 2006. Accepted for publication October 18, 2006.

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¹ This work was supported by Swiss National Science Foundation Grants 3100-63381/2 and 3100-063381/3. The support of the OPO-Stiftung Zurich to R.C. is gratefully acknowledged.

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⁴ Abbreviations used in this paper: Trx, thioredoxins; AE, atopic eczema; SPT, skin-prick test; APT, atopy patch test; EU, ELISA unit.

Table I. Primers used for the amplification of thioredoxins

Allergen	Accession No.	5'-Primer ^a	3'-Primer ^a
Human Trx	X77584	5'- <u>cgggatcc</u> atggtgaagcagatcgagagc-3'	5'- <u>cccaagctt</u> agactaatcattatggtggct tccagcttttcc-3'
Mala s 13	AJ937746	5'- <u>cgcgga</u> tccgtgcaagtgattttcttcgtagc-3'	5'- <u>cccaagctt</u> agggccgagtgctgggtg-3'
Asp f 28	AJ937744	5'- <u>cgcgga</u> tccatgtcccatggcaagttatag-3'	5'- <u>cccaagctt</u> aagcagaatactcttaatcatctcc-3'
Asp f 29	AJ937745	5'- <u>cgcgga</u> tccatgtctcacaacgtcgagaag-3'	5'- <u>cccaagctt</u> aagcaacgtgcgctttgatag-3'

^a The BamHI and HindIII restriction sites on the 5' and 3' primers, respectively, are underlined.

was those of the major birch pollen allergen Bet v 1 and the Fab of the murine mAb BV16, directly confirming the conformational nature of B cell epitopes involved in allergy (15).

In this work, an alternative approach was used to predict putative B cell epitopes of homologous proteins involved in IgE-mediated cross-reactivity. They can be identified by determining shared features on the level of primary and tertiary structure. The three-dimensional structure of *M. sympodialis* Trx (Mala s 13), a novel allergen, was determined at 1.4-Å resolution by x-ray diffraction analysis and compared to the solved structure of human Trx (16) to define solvent-accessible residues shared by the two crystal structures potentially involved in IgE-mediated cross-reactivity among Trx.

Materials and Methods

Cloning, protein expression, and purification of Trx

The novel *M. sympodialis* and *A. fumigatus* Trx cDNAs were amplified by standard PCR from clones isolated by phage surface display (7) using the primers listed in Table I and Pfu Turbo DNA Polymerase (Stratagene). They were formally termed Mala s 13, Asp f 28, and Asp f 29 according to the recommendations of the International Allergen Nomenclature Committee (17). Human TRX was amplified from a commercial human lymphoma U937 lung cDNA library (Stratagene) and sequence-derived primers (Table I). PCR amplification products were subcloned as BamHI/HindIII fragments into pQE 30 (Qiagen) and transformed into *Escherichia coli* M15 electrocompetent cells followed by DNA sequence verification.

To produce the corresponding His-tagged recombinant proteins, *E. coli* M15 cells containing the correct constructs were grown at 37°C in Terrific Broth medium to an OD₆₀₀ of 1.0, induced with 1 mM isopropyl β-D-thiogalactoside, incubated at 25°C for 24 h, harvested by centrifugation at 6000 × g for 10 min at 4°C, and stored at -20°C. The cell pellet was resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 50 mM imidazole (pH 8.0)) and lysed by French press. The insoluble material was removed by centrifugation at 20,000 × g (20 min, 4°C).

The His-tagged recombinant proteins were purified by nickel-affinity chromatography using 5 ml of HiTrap Chelating HP columns (Amersham Biosciences) according to the manufacturer's recommendations. Bound proteins were eluted in a linear buffer gradient (50–250 mM imidazole, 50 mM NaH₂PO₄, and 300 mM NaCl (pH 8.0)). The eluted proteins were dialyzed against distilled H₂O, tested for correct size and purity by SDS-PAGE, aliquoted, and stored at -20°C until use.

For crystallization of Mala s 13, a thrombin-cleavable His-tagged protein was designed. The Mala s 13 cDNA was amplified from the original clone by PCR with the primers 5' BamHI, 5'-CCGCGGATCCGTGCAAGTGATTTCTTC-3' and 3' HindIII, 5'-GCCCAAGCTTTTAGGCCGAGTGCTGG-3' using the Pfu Turbo DNA Polymerase (2.5 U/μl; Stratagene). The PCR product was digested with BamHI and HindIII, cleaned with a QIAquick PCR purification kit (Qiagen), and ligated into a modified pQE32 vector containing an N-terminal His₆ tag followed by a thrombin cleavage site (HHHHHHLVPRGS), where GS corresponds to the BamHI site. After the cleavage site, the coding sequence started with the second amino acid (valine) of the mature protein. The ligation mixture was transformed into *E. coli* strain M15 and the sequence of picked clones containing inserts of the correct size was verified by DNA sequencing. A correct clone was used to produce and purify His-tagged protein processed as before. Because thrombin cleavage of the N-terminal His tag was not functional under various conditions, the protein was processed and crystallized uncut. After purification by gel filtration on a Superdex 75 column, fast protein liquid chromatography (Pharmacia Biotech) equilibrated with

100 mM NaCl and 50 mM Tris (pH 7.5), Mala s 13 was diluted 1/10 in H₂O and concentrated to 10 mg ml⁻¹.

Enzymatic activity measurements

Enzymatic activity of the recombinant Trx was assessed by measuring the catalytic reduction of insulin as described previously (18). In brief, the reaction mixtures contained 0.1 mmol l⁻¹ potassium phosphate (pH 6.5), 2 mmol l⁻¹ EDTA, 0.13 mmol l⁻¹ bovine insulin (Sigma-Aldrich), 0.33 mmol l⁻¹ DTT, and 3.9 mmol l⁻¹ of the respective Trx. The precipitation of insulin was monitored with a Uvikon SL spectrophotometer (Bio-Tek Instruments) at a wavelength of 650 nm.

IgE immunoassays, inhibition ELISA, and immunoblots

IgE binding to recombinant Trx was determined by a standard direct solid-phase ELISA in polystyrene microtiter plates (Maxisorp; Nunc) coated and processed as described elsewhere (19). Results were expressed as ELISA units per ml (EU/ml) calibrated against the absorbency of an in-house reference standard arbitrarily defined as 100 EU/ml for each allergen tested (19). Inhibition ELISA was performed using 1/10 diluted patient's sera and BSA as a negative control as reported elsewhere (20).

For Western blot, 1 μg of recombinant protein was subjected to SDS-PAGE (NuPAGE, 4–12% Bis-Tris; Invitrogen Life Technologies), electrotransferred onto Hybond ELC membranes (Amersham Biosciences), and processed as described previously (20).

Subjects, routine assessments, and skin tests

Sera from 40 patients suffering from AE, diagnosed according to the criteria of Hanifin and Rajka (21) and sensitized to *M. sympodialis*, were selected according to clinical history and immediate skin reactivity to fungal extract and analyzed along with the sera of 10 healthy controls. Allergen-specific IgE to extract was determined by ImmunoCAPs m70 (*M. sympodialis*) using the Pharmacia CAP system (Pharmacia Biotech) according to the package insert. Routine skin-prick tests (SPT) and atopy patch tests (APT) were performed with an in-house *M. sympodialis* (ATCC strain 42132) extract prepared as described previously (22). SPT and APT with Mala s 13 and human Trx were performed as described elsewhere (23). The study protocol was conducted according to a clinical protocol approved by the ethical committee of the University of Zurich, and all participants gave written informed consent after a full explanation of the procedure given individually before testing.

Crystallization and data collection

Crystallization was performed using the hanging drop vapor diffusion method at 23°C. Four microliters of protein solution (10 mg/ml) was mixed with 2 μl of reservoir solution (1.8 M ammonium sulfate, 3% PEG 400, and 0.1 M imidazole (pH 7.0)). The drop was equilibrated against 500 μl of reservoir solution. After 2 wk, small crystals grew, which reached a size of ~250 × 60 × 60 μm after 2 mo. Crystals were cryoprotected by soaking stepwise for 1 min in reservoir solution complemented with increasing amounts of ethylene glycol (5, 10, and 15%). The crystals were flash-cooled in a stream of gaseous nitrogen and a dataset was collected to a 1.4-Å resolution on the synchrotron beamline X06SA at Swiss Light Source (Villigen/CH) at 100 K. Data were processed and scaled with Denzo and Scalepack of the HKL program package (24). The crystals belong to the monoclinic space group P2₁ with cell parameters a = 37.50 Å, b = 51.99 Å, c = 53.02 Å, and β = 99.49° (Table II).

Structure determination and refinement

The structure was solved by molecular replacement using MOLREP (25). A polyalanine model of *Chlamydomonas reinhardtii* Trx H (Brookhaven Protein Data Bank (PDB) code 1EP7; Ref. 26) served as search model.

Table II. Data collection and refinement statistics^a

Data Collection	
Wavelength (Å)	1.00802
Unit cell dimensions a, b, c (Å)	37.50, 51.99, 53.02
a, b, g (°)	90.00, 99.49, 90.00
Resolution (Å)	37.0-1.41 (1.43-1.41)
Unique reflections	38,712
Redundancy	4.4
Completeness (%)	99.6 (93.2)
Rsym (%)	4.5 (24.7)
Average I/s	18.9 (3.4)
Refinement Statistics	
Resolution (Å)	37.0-1.41
No. of reflections (working/test)	36,757/1938
No. of atoms	2011
Rcryst (%)	14.0
Rfree (%) ^b	16.8
Mean B factor (Å ²): All atoms	18.5
Main chain atoms (A/B)	15.3/16.0
Side chain atoms (A/B)	19.4/19.4
Solvent atoms	29.8
Rmsd bond lengths (Å)	0.016
Rmsd bond angles (°)	1.45

^a Number in parentheses are for the highest resolution shell.^b Rfree was calculated using a test set of 5%.

Two molecules were located in the asymmetric unit yielding a Matthews coefficient (V_M) of 1.91 Å³/Da and a corresponding solvent content of 35.7%. An initial rigid body refinement using REFMAC (27) as implemented in the CCP4 program suite (28) resulted in an R and R_{free} of 52 and 53%, respectively. Further refinements were performed with REFMAC, manual rebuilding, and correction with XtalView (29). Initially, noncrystallographic symmetry restraints were used, which were stepwise released and finally omitted. After a few refinement cycles, positive peaks in the difference electron density indicated well-ordered His tags in both molecules, which were modeled accordingly. A total of 164 water molecules was introduced using an automated refinement procedure (30). Final rounds of refinement were conducted with individual anisotropic B factors. The side chain of Met⁷⁴ in chain A, as well as Gln³, Lys⁵⁶, and Ser⁶³ in chain B, were modeled as double conformations with occupancies of 0.6/0.4. During the final refinement cycles, the $F_o - F_c$ difference in electron density clearly showed disordering in the residue ranges 30–37 and 73–75,

respectively, of chain B with positive and negative peaks, indicating an alternate peptide conformation. Therefore, the main and side chains of these two ranges were modeled as double conformations with occupancies of 0.6 and 0.4 for the original and alternate conformation, respectively. Statistics from data collection and refinement are provided in Table II. The stereochemical quality of the final model was assessed with PROCHECK (31) and WHATCHECK (32). Anisotropic validation was done with PARVATI (33). Secondary structure elements were assigned automatically with DSSP (34). Coordinates and structure factors have been deposited in the Protein Data Bank (PDB code 2J23).

Calculation of the solvent-accessible area

Solvent-accessible surface areas were calculated from molecule A of the solved Mala s 13 structure as well as from the molecule of the oxidized human Trx structure (PDB code 1ERU; Ref. 16). Calculations were performed with the program nAccess (35), an implementation of the Lee and Richards solvent accessibility algorithm (36), using a probe radius of 1.4 Å with a slice width of 0.01 Å and omitting all water molecules. The relative residue accessibility is the ratio of the accessible area of a residue in the model to the accessible area of that residue in an extended Ala-X-Ala tripeptide.

Results

Molecular cloning of Mala s 13, Asp f 28, Asp f 29, and production of recombinant Trx

Screening of *M. sympodialis* and *A. fumigatus* cDNA libraries displayed on phage surface yielded vast varieties of clones carrying inserts encoding putative IgE-binding proteins (5, 6). Among these, one *M. sympodialis* and two *A. fumigatus* clones coded for cDNAs sharing high sequence identity with several Trx at primary structure level (Fig. 1). The cDNAs contained open reading frames of 318, 327, and 333 bp, coding for proteins of 105, 108, and 110 aa with the conserved active site residues of Trx, and calculated molecular masses of 11.46, 11.94, and 11.98 kDa. The new allergens were formally termed Mala s 13, Asp f 28, and Asp f 29 according to the recommendations of the allergen nomenclature committee (17). The sequences are available from the National Center for Biotechnology Information database under accession numbers AJ937746, AJ937744, and AJ937745, respectively. PCR-amplified DNAs encoding full-length Mala s 13, Asp f 28, Asp f 29, and human Trx were ligated into pQE 30, expressed as N-terminal hexahistidine-tagged proteins in *E. coli*, purified by

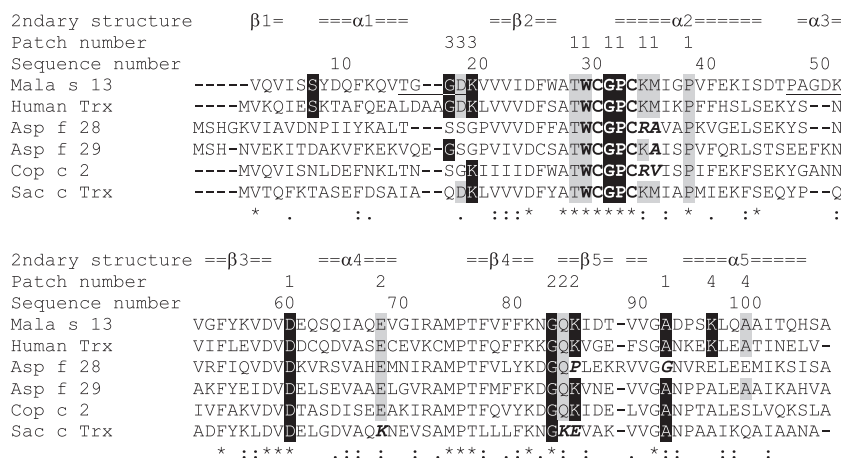


FIGURE 1. Structure alignment of Mala s 13 and human Trx combined with a sequence alignment of *A. fumigatus* Trx (Asp f 28 and Asp f 29), *C. comatus* Trx (Cop c 2), and *S. cerevisiae* Trx (Sac c Trx). The top line denotes the secondary structure of Mala s 13 assigned by DSSP (34), the second line shows the number and position of the conserved patch residue, and the third line shows the sequence numbering of Mala s 13. Asterisks, identical, colons strongly similar and periods weakly similar amino acids in all sequences. Active site residues are in bold. The two loops, which adopt a different conformation in Mala s 13 compared with human Trx, are underlined. Residues that are identical and at least 50% or between 30 and 50% solvent exposed in Mala s 13 and human Trx are shown on a gray or black background, respectively. Residues, which are also conserved in Asp f 28, Asp f 29, Cop c 2, or Sac c Trx, are colored correspondingly. Residues of these Trx corresponding to point mutations with respect to patch 1 and 2 residues of Mala s 13 and human Trx are depicted in bold italics.

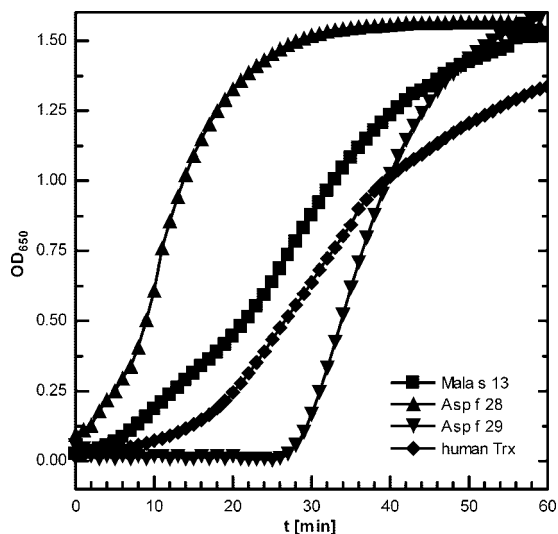


FIGURE 2. Trx-catalyzed reduction of bovine insulin by DTT.

Ni²⁺-NTA affinity chromatography, and analyzed on 12% SDS-PAGE. The His-tagged proteins migrated as a single band in good agreement with the predicted size (data not shown). The activity of the proteins was demonstrated by the enzymatic reduction of insulin (Fig. 2), indicating native-like folding, a prerequisite for IgE binding.

Allergenicity of the recombinant Trx

IgE binding of the Trx was surveyed by ELISA using sera of 40 patients with AE sensitized to *M. sympodialis* and 10 sera of healthy individuals (Fig. 3). Sera were considered positive when the calculated EU/ml values were >3-fold higher than the mean EU/ml value of the healthy controls for the respective allergen. IgE binding was also confirmed by Western blot analysis (data not shown).

To explore whether the IgE of patients sensitized to Mala s 13 cross-reacts with different Thx, we performed competition ELISA.

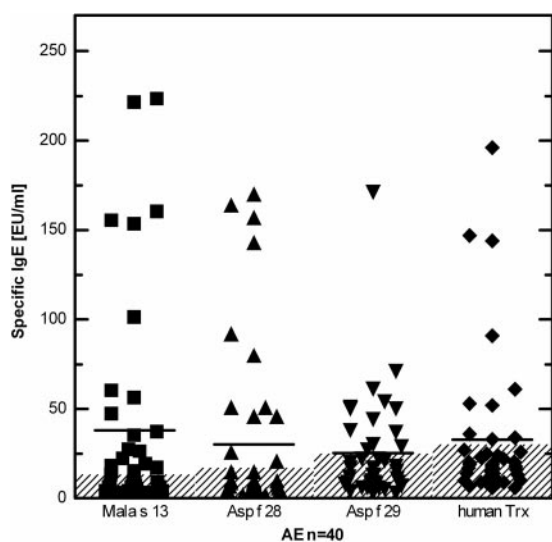


FIGURE 3. IgE binding to allergens in ELISA. Ag-specific ELISA is compared with the binding of a reference serum arbitrarily assigned to 100 EU/ml (19) in 40 patients suffering from AE and 10 healthy individuals. Mean values are indicated by lines. The hatched areas represent the 3-fold value of the mean EU/ml value of 10 healthy controls for the respective allergen.

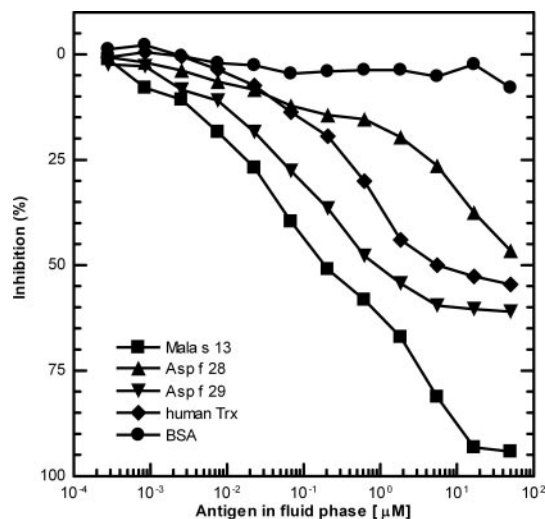


FIGURE 4. Inhibition ELISA. Competitive inhibition of IgE binding to recombinant Mala s 13 coated on a solid phase. Pooled serum from *M. sympodialis*-sensitized patients was preincubated with increasing amounts of recombinant Mala s 13, Asp f 28, or Asp f 29, human Trx and BSA as a negative control. Preincubated serum samples were transferred to wells coated with Mala s 13, and residual IgE binding was analyzed by ELISA.

Distinct cross-reactivity was observed between Mala s 13 and Asp f 29, which share 49.5% sequence identity, whereas cross-reactivity between Mala s 13 and Asp f 28 (41.9% sequence identity) was less pronounced (Fig. 4). cross-reactivity between Mala s 13 and human Trx (45% identity) was intermediate, and no inhibition was observed using BSA as a negative control (Fig. 4). A tentative explanation for the observed differences in cross-reactivity between the structures can be derived from a detailed analysis of potential cross-reactive epitopes predicted from analyses of the Mala s 13 crystal structure (see below).

The clinical relevance of the cross-reactivity between Mala s 13 and human Trx was assessed by SPT and APT as described previously (23). Five patients with and five without allergen-specific IgE to Mala s 13 sensitized to *M. sympodialis* and five healthy controls were tested. None of the individuals lacking detectable serum IgE Abs to Mala s 13 reacted against Mala s 13 or human Trx, and all individuals who had positive Mala s 13-specific IgE serum levels in ELISA reacted positively to skin challenges with the recombinant proteins. Unfortunately, it was not possible to perform APT in all patients with acute dermatitis showing high Mala s 13-specific IgE serum levels, because of the lack of unaffected skin areas available for testing. However, the results presented in Table III clearly show a highly specific skin test reactivity of both fungal and human enzyme.

The overall structure of Mala s 13

The crystal structure of Mala s 13 was solved at 1.4-Å resolution by molecular replacement and refined to a crystallographic *R* factor of 14.0% (free *R* factor 16.8%). The final parameters of refinement are given in Table II. A total of 94.2% of the non-glycine and nonproline residues have main chain dihedral angles in the most favored regions of the Ramachandran plot (37), with the remaining ones located in the additional allowed regions. There are two independent molecules per asymmetric unit, designated A and B. The final model consists of all amino acids of both independent monomers (aa 2–105) and 164 water molecules. In molecule A, which was used for the calculation of

Table III. Induction of immediate and late skin reactions by Mala s 13 in atopic eczema

No.	Sex ^a	Age (years)	Scorad	Total IgE (kU/L)	m70 ^b IgE (kU/L)	Mala s 13 IgE (EU/ml)	SPT ^c		APT ^d	
							Mala s 13	hTrx	Mala s 13	hTrx
1	M	28	13.0	921	7.21	84	+	+	+	+
2	M	28	43.0	278	16.20	98	+	+	+	+
3	F	53	13.8	195	21.38	156	+	+	+	+
4	F	45	19.8	308	10.40	75	+	+	+	+
5	M	32	54.0	2750	18.70	141	+	+	+	+
6	F	42	21.0	782	<0.35	<10	—	—	—	—
7	F	44	18.0	1180	<0.35	<10	—	—	—	—
8	M	41	25.0	830	<0.35	<10	—	—	—	—
9	M	19	9.9	402	<0.35	<10	—	—	—	—
10	M	27	18.4	267	<0.35	<10	—	—	—	—
11 ^e	F	31	<5	26	<0.35	<10	—	—	—	—
12 ^e	M	47	<5	72	<0.35	<10	—	—	—	—
13 ^e	M	52	<5	88	<0.35	<10	—	—	—	—
14 ^e	F	26	<5	75	<0.35	<10	—	—	—	—
15 ^e	M	32	<5	35	<0.35	<10	—	—	—	—

^a M, Male; F, female.^b *M. sympodialis* ImmunoCAPm70 (Pharmacia).^c A wheal diameter >3 mm surrounded by erythema in SPT was considered positive. Plus and minus signs denote positive and negative, respectively.^d APT was considered positive when signs of induction of an eczematous reaction were visible after 48 h. Plus and minus signs denote positive and negative, respectively.^e Healthy controls.

the solvent-accessible surface, all the side chains of the amino acids on the surface are well defined, except of Lys⁵¹, Gln⁶⁷, and Arg⁷². These side chains are solvent exposed and, thus, freely movable. Interestingly, the His tags of both molecules are visible in the electron density from the third histidine onward, which is quite rare. They are sandwiched between two neighboring, symmetry-related molecules and are thereby stabilized. Both monomers show the typical Trx fold, consisting of a five-stranded β sheet forming a hydrophobic core surrounded by four α helices (Fig. 5) with an additional small α helix formed by residues Ala⁴⁸-Lys⁵¹.

In chain A, the conserved active site amino acids Trp²⁹-Cys-Gly-Pro-Cys are well defined in the electron density. They link the second β strand to the second α helix, with Pro-Cys forming the first turn of the helix. The cysteines are in the oxidized form, as expected under oxidizing crystallization conditions. The sulfur atoms are very well defined in the density map and were, thus, re-

finned without a disulfide bond restraint resulting in a bond length of 2.29 Å and a difference electron density without any peaks around the sulfur atoms. In chain B, there is some disorder in the active site residues and in the first half of the following α helix (aa Cys³⁰-Gly³⁷) as well as in the residue range Ala⁷³-Pro⁷⁵. These two ranges, which lie in the vicinity of each other, were thus modeled in a double conformation. The cysteines are also present in the oxidized form.

The two independent molecules form a crystallographic dimer related by a noncrystallographic 2-fold axis. The first β strand of each molecule is hydrogen bonded to each other, which results in an extended β sheet between the two molecules consisting of 10 strands. The dimer interface buries a surface area of 1000 Å² on each molecule. Without the His tags, the contact interface drops to 713 Å², thus the His tags contribute substantially to the monomer-monomer interaction. The arrangement of the dimer is different from the natural covalent dimer observed in human Trx. The human dimer results from an intermolecular disulfide bond via the nonconserved residue Cys⁷³ of each monomer and is supposed to have a regulatory function, because the active site becomes buried and thus inactive on dimer formation (16). In contrast, the Mala s 13 dimer is not affecting the active site and is most probably of crystallographic nature only. Trx are known to be redox active in their monomeric form. In solution, Mala s 13 behaves as a monomer as demonstrated in a gel filtration experiment with four marker proteins on a Superdex 75 column, resulting in an elution volume of 13.01 ml and a calibrated mass of 12.8 kDa, which is well in agreement with the calculated His-tagged monomer mass of 13.3 kDa.

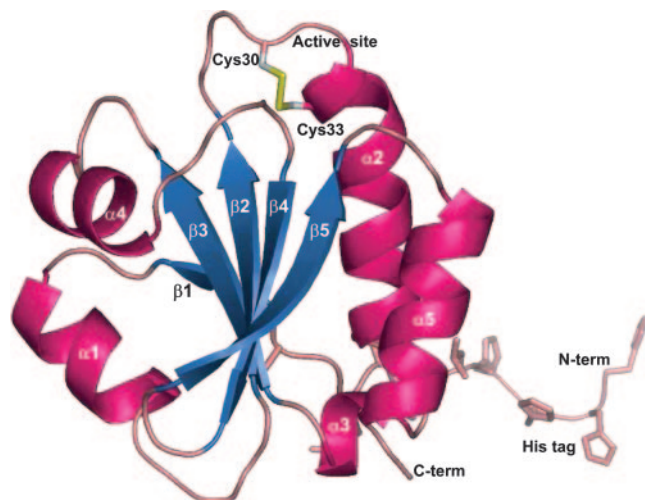
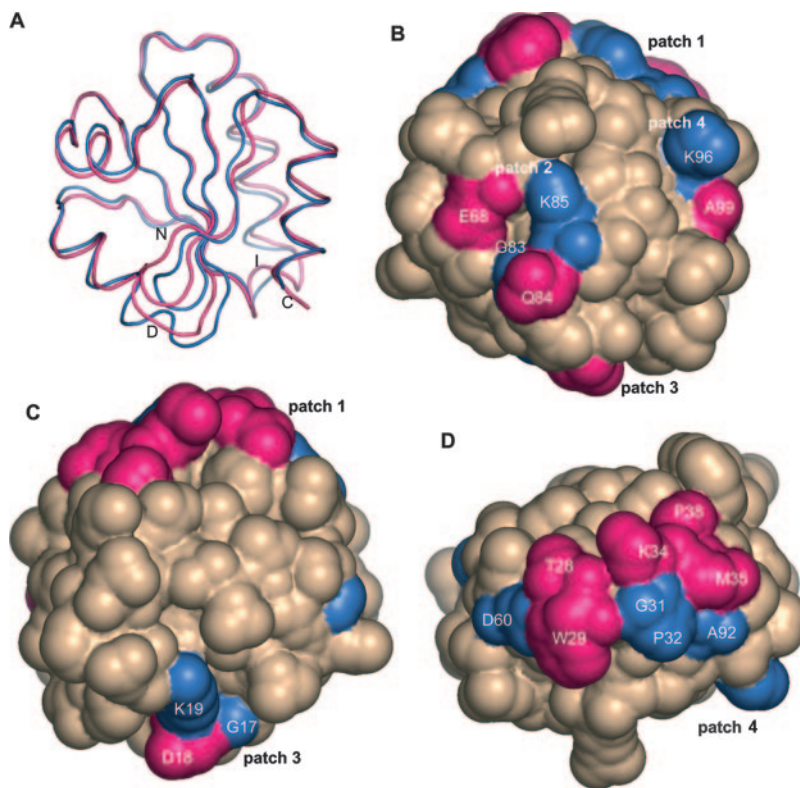


FIGURE 5. Cartoon representation of Mala s 13. The overall Mala s 13-fold consists of a five-stranded β sheet forming a hydrophobic core surrounded by five α helices. The side chains of the active site cysteines and the His tag are shown as a capped stick model.

Superposition of Mala s 13 on human Trx reveals putative IgE-binding surface areas

Mala s 13 shares 45% sequence identity with human Trx. Superposition of the backbones of the solved Mala s 13 structure with the oxidized human Trx (PDB code 1ERU; Ref. 16) revealed a high structural similarity with a root mean square deviation of 1.11 Å for all C α atoms (Fig. 6A) with two conformational differences. First, a deletion of two amino acids between Gly¹⁶ and Gly¹⁷ of Mala s 13 shortens the end of the first α helix. Second, an insertion

FIGURE 6. Putative solvent-exposed IgE-binding residues. **A**, Superposition of Mala s 13 (pink) on human Trx (blue, PDB code 1ERU) reveals large structural similarities with two minor deviations. The first α helix is shortened due to a deletion, D. There is an additional small α helix due to an insert, I. The N and C termini are designated N and C, respectively. **B–D**, Solvent-accessible surfaces of Mala s 13 showing putative IgE-binding residues. Amino acids that are identical and at least 50% or between 30 and 50% solvent-exposed in Mala s 13 and human Trx are shown in pink and blue, respectively. **B**, The front view reveals four conserved surface patches. Patch 2 comprises 4 aa (Glu⁶⁸ and Gly⁸³-Lys⁸⁵) covering a surface area of 350 Å² and likely accounts for a B cell epitope. Patch 4 (Lys⁹⁶, Ala⁹⁹) is most probably too small to form an IgE-binding epitope. Scale and view are as in **A**. **C**, The backside is virtually devoid of conserved residues. Patch 3 (Gly¹⁷-Lys¹⁹) on the bottom is rather small and lies in a region with structural deviation. Therefore, it probably does not account for an IgE-binding epitope. The view is as in **A**, but rotated by 180° about the y-axis. **D**, Patch 1 consists of residues forming and surrounding the active site. It fulfills the criteria for a putative Ag-Ab interaction quite well and is also conserved among other IgE-binding Trx. The view is as in **A**, but rotated by 110° about the x-axis.



of two residues (Gly⁴⁹ and Asp⁵⁰) leads to an additional small α helix after the second helix of Mala s 13.

As shown before, Mala s 13 and human Trx show cross-reactivity leading to IgE autoreactivity in patients suffering from AE. Therefore, these proteins must share common IgE-binding epitopes. Only those residues that are at least partly exposed to solvent can contribute to Ag-Ab interactions in native proteins. Thus, solvent-accessible residues conserved in both proteins are potentially involved in the IgE-mediated cross-reactivity experimentally confirmed in vitro (Fig. 4) and in vivo (Table III). A sequence alignment of Mala s 13 and human Trx shows that a total of 48 of the 105 aligned residues are identical (Fig. 1). Of the 48 identical amino acids, 19 are at least 30% and 9 of them at least 50% solvent exposed in both structures.

These identical, solvent-exposed residues in Mala s 13 and human Trx were mapped on the solvent-accessible surface of Mala s 13 (Fig. 6, B–D). The figures reveal conserved, contiguous patches, which might represent conformational, cross-reactive IgE-binding epitopes. Fig. 6B shows four patches, including a large one on top (patch 1), one in the middle (patch 2), and one on the bottom (patch 3). A fourth patch of only 2 aa (K96 and A99) is probably too small to contribute to IgE binding. Patch 1 consists of amino acids that form the active site and the beginning of the following α helix (Thr²⁸, Trp²⁹, Gly³¹, Pro³², Lys³⁴, Met³⁵, and Pro³⁸) lying all on the same peptide stretch (Fig. 6D). There are two more residues contributing to this patch that are situated on sequentially more distant loops (Asp⁶⁰ and Ala⁹²). The whole patch covers a solvent-accessible surface area of 846 Å² and fulfills the criteria for a putative Ag-Ab interaction surface quite well.

Patch 2 comprises 4 aa distributed on α helix 4 and the beginning of β sheet 5 (Glu⁶⁸ and Gly⁸³-Lys⁸⁵), which cover a surface area of 350 Å². Together with neighboring amino acids, which are less solvent-exposed or which are not identical, but strongly similar in both proteins, the conserved patch forms an area large enough to accommodate a cross-reactive IgE Ab. Patch 3 is formed

by amino acids of the loop after the first α helix (Gly¹⁷-Lys¹⁹) and covers an area of 246 Å² (Fig. 6C) but lies in a region with structural deviation between Mala s 13 and human Trx in which there are two additional amino acids before Gly¹⁷ of the GDK-conserved motif (Fig. 1). Therefore, it is unlikely to account for cross-reactivity. To strengthen our hypotheses, the sequences of further Trx shown to be cross-reactive have been included to identify conserved residues. The structures of these four cross-reactive Trx (Asp f 28, Asp f 29, Cop c 2, and *Saccharomyces cerevisiae* Trx) are not known, but they can be compared on a sequential level to Mala s 13 and human Trx (Fig. 1). The alignment shows that patch 1 is highly conserved among all proteins. Five amino acids are fully conserved, whereas in three positions corresponding to Lys³⁴, Met³⁵, and Ala⁹² natural mutations are observed. Within the Asp f 29 sequence, alanine replaces Met³⁵ of Mala s 13. Mutations at positions 34, 35, and 92 occur in the Asp f 28 sequence, namely, Lys³⁴/Arg, Met³⁵/Ala, and Ala⁹²/Gly. The alignment shows that amino acid exchanges occur also at position 34 (Lys³⁴/Arg) and position 35 (Met³⁵/Val) of Cop c 2, whereas patch 1 amino acids are fully conserved in the Sac c Trx sequence (Fig. 1). The high conservation of this patch situated around the active site indicates that it might represent a dominant IgE-binding epitope. Patch 2 is also strongly conserved among the proteins with the exception of Sac c Trx. A single mutation is present in Asp f 28 where Lys85 is substituted by proline. Also in patch 2, the rather high conservation of the amino acids support the assumption that it might account for a second overall cross-reactive epitope. Allergens must carry at least two IgE-binding epitopes (or copies of a repetitive epitope) to cross-link IgE Abs and trigger hypersensitivity reactions clearly demonstrated to occur by in vivo testing of the Trx investigated (Table III). Therefore, the two suggested epitopes could indeed be responsible for the overall cross-reactivity of Trx. The ELISA results showing that Asp f 28 is poorly cross-reactive corroborate this hypothesis. In fact, Asp f 28 is, among the experimentally investigated Trx, the one with the highest degree of

sequence variability within path 1 and 2 and also the weakest IgE-binding protein. Patches 3 and 4 are hardly conserved among the six Trx, further supporting the assumption that these surface areas are not accounting for cross-reactivity. Thus, amino acids forming patches 1 and 2 are excellent candidates to study their contribution to cross-reactivity by further site-directed mutagenesis crystallization of the mutants and solution of the three-dimensional structures, followed by determination of their IgE-binding capacity.

Discussion

Pan-allergens are cross-reactive structures involving phylogenetically conserved proteins present in unrelated allergenic sources. Prominent examples of them are profilins (38), lipid transfer proteins (39), and cyclophilins (20). They are responsible for often-observed clinical syndromes like birch-mugwort-celery syndrome (40), oral allergy syndromes (41), and AE/dermatitis syndrome (42). Perhaps the most interesting class of pan-allergens includes environmental allergens sharing structural homology to self-Ags. These structures, including manganese superoxide dismutase, acidic ribosomal P₂ proteins, cyclophilins, and profilins (9, 11, 12, 20, 38), have been implicated in exacerbation and/or perpetuation of severe atopic disorders. In contrast to allergenicity, which is an intrinsic property of many different molecular structures, Ab-mediated cross-reactivity among homologous proteins is largely determined by common structural features shared between protein families (13). However, not many crystal structures of allergens in general and of cross-reactive allergens in particular are available that would allow studying Ab-mediated cross-reactivity in detail (13). In this study, we describe cross-reactivity and crystal structure of Mala s 13, a novel member of a new pan-allergen family. Notably, cross-reactivity between fungal and human Trx can be demonstrated in vitro (Fig. 3) as well as in vivo (Table III). The crystal structure of Mala s 13 was determined at 1.4 Å and compared with the structure of human Trx, which had been determined at 2.1 Å resolution (16). To define surface regions potentially involved in IgE-mediated cross-reactivity, we determined shared features on the level of primary and tertiary structure. Although the fungal and the human enzymes share 48 identical amino acids (Fig. 1), only 19 of them are >30% and only 9 are >50% solvent exposed in both structures and, therefore, likely to be accessible for Ag-Ab interactions. They are scattered over the whole sequence (Fig. 1) and become clustered over the surface, forming two relevant patches covering solvent-accessible surface areas of 846 Å² and 350 Å², respectively, potentially forming conformational B cell epitopes (Fig. 6, B and D). Patch 1 fulfils the criteria for a putative Ag-Ab interaction regarding distribution and number of involved residues and total surface area of the patch. The known B cell epitopes derived from cocrystallization experiments between Ag and Ag-specific Fabs occupy a buried surface in the range of 540–890 Å² and are formed by 15–22 aa residues on different surface loops. Patch 2 with a smaller surface could, along with neighboring homologous amino acids conserved in both proteins (e.g., I71, R72, I86), form an area large enough to accommodate a cross-reactive epitope. There is a third patch of 246 Å² surface which lies in a region with structural deviation between Mala s 13 and human Trx (Fig. 6A) due to a two-amino acid deletion in Mala s 13 compared with human Trx after Gly¹⁶ (Fig. 1) and, thus, probably does not account for a cross-reactive epitope, whereas patch 4, composed of only two amino acids (K96 and A99), is probably too small for an Ag-Ab interaction. These patches are not well conserved in Asp f 28 and Asp f 29, two new allergenic Trx of *A. fumigatus* shown to cross-react with Mala s 13 in this work, and the other Trx further supporting the argument that this region is not contributing to cross-reactivity. Although the structures of

Asp f 28 and Asp f 29 are not known, they can be compared on a sequential level to Mala s 13 and human Trx under the assumption of fold conservation within the Trx family. The alignment (Fig. 1) shows that the majority of the amino acids of patch 1 are highly conserved, indicating a putative epitope involved in cross-reactivity. Patch 2 is also highly conserved among the structures, including the neighboring homologous amino acids and most likely defines a second epitope required to cross-link IgE Abs to trigger the hypersensitivity reactions reported on Table III. The naturally occurring mutations within the two patches present in the experimentally tested Trx and differences in cross-reactivity showing that the protein with the highest mutation rate (Asp f 28) corresponds to the weakest IgE-binding protein corroborate the involvement of the two defined surface areas in cross-reactivity. However, at this stage, assigning a role to every single amino acid of the two patches in the interaction with IgE is not possible. Cross-reactivity between Trx is not complete and is around 50% as shown in Fig. 4. The most probable explanation for this observation is that exposure to environmental Trx elicits a vast variety of Abs specific for different B cell epitopes during the time course of a polyclonal IgE response, because the whole surface of a protein is potentially immunogenic (13). Only identical or highly homologous amino acid residues exposed on the surface of two homologous structures can account for Ab-mediated cross-reactivity. As shown by comparison of the Mala s 13 and human Trx structures, the conserved areas forming patches able to accommodate putative cross-reactive B cell epitopes are quite limited. The most exciting aspect of the cross-reactivity between environmental allergens and self-Ags demonstrated here for Trx and elsewhere for other homologous molecular structures (9–12, 20, 43) consists in their ability to influence the pathogenesis of severe atopic diseases (23). The availability of the crystal structures of allergen/self-Ag pairs provides structural information for the modification of the cross-reactive areas by site-directed mutagenesis. Molecules depleted from amino acids involved in cross-reactivity have a high therapeutic potential (44) and will allow detailed studies of the role played by humoral and cell-mediated autoreactivity on pathogenic processes involved in allergic diseases.

Acknowledgments

We thank D. Kloer for his great support in x-ray crystallography and T. Tomizaki from the Swiss Light Source for his technical help. This work was performed at the Swiss Light Source, Paul Scherrer Institut, Villigen, Switzerland. We are grateful to D. Kostrewa, M. Grütter, and P. Mittl for their support and the opportunity to collect test data on their home sources.

Disclosures

The authors have no financial conflict of interest.

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