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

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

Andreas Limacher, Andreas G. Glaser, Christa Meier, Peter Schmid-Grendelmeier, Sabine Zeller, Leonardo Scapozza and Reto Crameri

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 Ca 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) 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 WGCPC. 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).

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2 A.L. and A.G.G. contributed equally to this work.

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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.

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was those of the major birch pollen allergen Bet v 1 and the Fab before cleavage of the N-terminal His tag was not functional under various conditions, the protein was processed and crystallized into E. coli M15 electrocompetent cells followed by DNA sequence verification.

### 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 pQE32 vector containing an N-terminal His6 tag followed by a thrombin cleavage site (HHHHHHLVPRGS), where GS corresponds to the 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 ligand was amplified by PCR with the primers listed in Table I and Pfu Turbo DNA Polymerase (Stratagene).

### Materials and Methods

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### Table I. Primers used for the amplification of thioredoxins

<table>
<thead>
<tr>
<th>Allergen</th>
<th>Accession No.</th>
<th>5'-Primer*</th>
<th>3'-Primer*</th>
</tr>
</thead>
<tbody>
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<td>Human Trx</td>
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<td>A937746</td>
<td>5'-cccgaagttctactactcttcctcgagacgacggtc-3'</td>
<td>5'-cccgaagttctactactcttcctcgagacgacggtc-3'</td>
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<tr>
<td>Asp f 28</td>
<td>A937744</td>
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<td>5'-cccgaagttctactactcttcctcgagacgacggtc-3'</td>
</tr>
<tr>
<td>Asp f 29</td>
<td>A937743</td>
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<td>5'-cccgaagttctactactcttcctcgagacgacggtc-3'</td>
</tr>
</tbody>
</table>

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

### 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 reaction 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 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 0.2–0.5 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 0.250 × 0.60 × 0.60 mm 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 IEP7; Ref. 26) served as search model.
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_M of 52 and 53%, respectively. Further refinements were performed with REFMAC, manual rebuilding, and correction with XtalView (29). Initially, noncrystalllographic 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.

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The side chain of Met74 in chain A, as well as Gln3, Lys56, and Ser63 in 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 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 rounds of refinement were conducted with individual anisotropic B factors.
Ni\textsuperscript{2+}-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 Trx, we performed competition ELISA.

Distinct cross-reactivity was observed between Mala s 13 and Asp f 28, which share 49.5% sequence identity, whereas cross-reactivity between Mala s 13 and Asp f 29 (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
Both monomers show the typical Trx fold, consisting of a five-stranded β sheet forming a hydrophobic core surrounded by five α helices. The side chains of the active site cysteines are well defined in the density map and were, thus, resolved in the oxidized form.

In chain A, the conserved active site amino acids Trp29-Cys30-Gly37-Pro38-Cys39 are well defined in the electron density. They link the first β strand to the second α helix, with Pro-Cys forming the second β strand and Cys-Pro-Cys 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, resolved 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.

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 Cx 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

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

<table>
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<th>No.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Scorad</th>
<th>Total IgE (kU/L)</th>
<th>m70 IgE (kU/L)</th>
<th>Mala s 13 IgE (EU/ml)</th>
<th>Mala s 13 hTrx</th>
<th>Mala s 13 hTrx</th>
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<td>1</td>
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<td>&lt;10</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<td>&lt;10</td>
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<td>-</td>
<td>-</td>
</tr>
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<td>14   F</td>
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<td>&lt;10</td>
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<td>-</td>
<td>-</td>
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<td>&lt;0.35</td>
<td>&lt;10</td>
<td>-</td>
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<td>-</td>
</tr>
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</table>

* M, Male; F, female.
* M. symподiatr. ImmunoCAPm70 (Pharmacia).
* A wheat diameter > 3 mm surrounded by erythema in SPT was considered positive. Plus and minus signs denote positive and negative, respectively.
* 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.
* Healthy controls.
of two residues (Gly\textsuperscript{40} and Asp\textsuperscript{50}) leads to an additional small \(\alpha\) helix after the second helix of Mala \textsubscript{s} 13.

As shown before, Mala \textsubscript{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 \textsubscript{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 \textsubscript{s} 13 and human Trx were mapped on the solvent-accessible surface of Mala \textsubscript{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 \(\alpha\) helix (Thr\textsuperscript{28}, Trp\textsuperscript{29}, Gly\textsuperscript{30}, Pro\textsuperscript{32}, Lys\textsuperscript{34}, Met\textsuperscript{35}, and Ala\textsuperscript{92}) 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 (Asp\textsuperscript{28} and Ala\textsuperscript{32}). The whole patch covers a solvent-accessible surface area of 846 Å\textsuperscript{2} and fulfills the criteria for a putative Ag-Ab interaction surface quite well.

Patch 2 comprises 4 aa distributed on \(\alpha\) helix 4 and the beginning of \(\beta\) sheet 5 (Glu\textsuperscript{40} and Gly\textsuperscript{53}–Lys\textsuperscript{55}), which cover a surface area of 350 Å\textsuperscript{2}. 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 \(\alpha\) helix (Gly\textsuperscript{17}–Lys\textsuperscript{19}) and covers an area of 246 Å\textsuperscript{2} (Fig. 6C) but lies in a region with structural deviation between Mala \textsubscript{s} 13 and human Trx in which there are two additional amino acids before Gly\textsuperscript{17} 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 \textsubscript{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 Lys\textsuperscript{34}, Met\textsuperscript{35}, and Ala\textsuperscript{92} natural mutations are observed. Within the Asp f 29 sequence, alanine replaces Met\textsuperscript{35} of Mala \textsubscript{s} 13. Mutations at positions 34, 35, and 92 occur in the Asp f 28 sequence, namely, Lys\textsuperscript{34}/Arg, Met\textsuperscript{35}/Ala, and Ala\textsuperscript{92}/Gly. The alignment shows that amino acid exchanges occur also at position 34 (Lys\textsuperscript{34}/Arg) and position 35 (Met\textsuperscript{35}/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 Lys\textsuperscript{85} 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 P0 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 fulfills 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 cocystallization 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 putative epitope involved in cross-reactivity. As shown by comparison of the Mala s 13 and human Trx structures, the conserved amino acids residues exposed on the surface of two homologous structures can account for Ab-mediated cross-reactivity. As shown by comparison of the Mal a 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.

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Disclosures

The authors have no financial conflict of interest.

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