

Molecular Characterization of a Fungal Cyclophilin Allergen Rhi o 2 and Elucidation of Antigenic Determinants Responsible for IgE-Cross reactivity

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ABSTRACT:

Cyclophilins are structurally conserved pan-allergens showing extensive cross-reactivity. So far, no precise information on cross-reactive IgE-epitopes of cyclophilins is available. Here, an 18 kDa IgE-reactive cyclophilin (Rhi o 2) was purified from *Rhizopus oryzae*, an indoor mold causing allergic sensitization. Based on LC-MS/MS-derived sequences of natural Rhi o 2, the full-length cDNA was cloned, and expressed as recombinant (r) allergen. Purified rRhi o 2 displayed IgE-reactivity and basophil degranulation with sera from all cyclophilin-positive patients. The melting curve of properly-folded rRhi o 2 showed partial refolding after heat denaturation. The allergen was monomeric functional peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. In IgE-inhibition assays, rRhi o 2 exhibited extensive cross-reactivity with various

other cyclophilins reported as allergens from diverse sources including its homologous human autoantigen. By generating a series of deletion mutants, a conserved 69 residues (N⁸¹-N¹⁴⁹) fragment at C-terminus of Rhi o 2 was identified as crucial for IgE-recognition and cross-reactivity. Grafting of the N⁸¹-N¹⁴⁹ fragment within the primary structure of yeast cyclophilin CPR1 by replacing its homologous sequence resulted in a hybrid molecule with structural folds similar to Rhi o 2. The IgE-reactivity and allergenic activity of the hybrid cyclophilin were greater than that of CPR1. Therefore, N⁸¹-N¹⁴⁹ fragment can be considered as the site of IgE recognition of Rhi o 2. Hence, Rhi o 2 serves as candidate antigen for the molecular diagnosis of mold allergy and determination of a major the cross-reactive IgE-epitope has clinical potential for the design of next-generation immunotherapeutics against cyclophilin-induced allergies.

The prevalence of IgE-associated allergy caused by fungal allergens is increasing worldwide at an alarming rate (1-4). Immunological cross-reactivity among taxonomically unrelated fungal, plant, and even animal species is often observed due to the presence of structurally conserved allergens such as cyclophilin, profilin, arginine kinase, and tropomyosin (5-8). The allergenic proteins showing a broad range of cross-reactivity are popularly known as pan-allergens. The similarity in sequence features and structural folds among the pan-allergens from diverse taxonomic groups gives rise to shared antigenicity, which in turn leads to immunological cross-reaction with patients' IgE-antibodies. In a particular group of patients, the cross-reactivity of pan-allergens can trigger an added IgE-mediated immune response that contributes to polysensitization leading to multiple symptoms like oral allergy syndrome (OAS), food allergy and respiratory allergy (9). Hence, the structural characterization of new cross-reactive allergens and their systematic inclusion into the list of pan-allergens are clinically relevant to elucidate the underlying mechanism of the disease.

Cyclophilins (or, rotamases) are a group of cyclosporine A binding chaperones that assist intracellular protein folding through *cis-trans* isomerization of prolyl bond and are therefore named as peptidyl-prolyl *cis-trans* isomerase or PPIase (10, 11). Cyclophilins are considered as a group of highly cross-reactive allergens that causes multivalent allergy in atopic individuals (12). Till now, 10 different cyclophilins have been reported from diverse sources such as carrot, birch pollen, dust mite, and *Aspergillus spp.*. Most impressive about these allergens is the presence of a high level of sequence conservation across different taxonomic groups leading to IgE-mediated cross-reactivity (12). An earlier report showed that due to molecular mimicry of B cell epitopes, the native cyclophilin of human origin could also act as autoallergen and cross-react with environmental cyclophilins in atopic dermatitis patients (13, 14). Asp f 11 from *Aspergillus fumigatus* (15), Bet v 7 from birch pollen (16), and Mala s 6 from skin colonizing yeast (17) are three major cyclophilin allergens showing a high frequency of sensitization worldwide. Hence, these cyclophilin allergens have potential to be

used in molecular diagnosis. It is noteworthy to mention that the plant cyclophilins are not yet properly investigated and deserve attention, in particular those from the grass pollen. In addition, cyclophilins from house dust mites could also be very important for tropical countries.

According to the epidemiological data, the fungal cyclophilins such as Mala s 6, and Asp f 11 are considered as most prevailing among various cyclophilin allergens and are therefore well characterized (12). In the present study, we report a new cyclophilin allergen (MW. 18 kDa) from *Rhizopus oryzae* (mucormycosis mold), which showed frequent IgE sensitization within the mold-allergic population and broad range of cross-reactivity with other reported cyclophilin allergens. This cyclophilin is designated as Rhi o 2 by WHO/IUIS (18). We also report the possible antigenic determinant of the Rhi o 2 structure through sequence deletion and epitope grafting experiments that may describe the molecular basis of cross-reactivity among cyclophilin sensitive patients.

RESULTS

Isolation of an IgE-antibody reactive cyclophilin from *R. oryzae* – To characterize the major allergen of *R. oryzae*, an 18 kDa IgE-binding protein was first purified by ion-exchange chromatography and was then identified as a cyclophilin by mass spectrometry. For purification, the resuspended pellet fraction obtained after 50% ammonium sulfate precipitation of the fungal crude protein extract was incubated with DEAE Sephacel gel. The resulting supernatant was found to have an enriched amount of the 18 kDa protein. The proteins in this supernatant were then fractionated in a carboxymethyl cation exchange column. The second fraction that eluted at around 450 mM NaCl concentration (Fr-2 in Fig 1a) was found to contain the desired 18 kDa protein with >90% purity. The purified protein displayed IgE reactivity when immunoblotted with sera from two mold allergic patients sensitized to *R. oryzae* (Fig 1b). In LC-MS/MS, the trypsinized protein was identified as a peptidyl-prolyl *cis-trans* isomerase from *Rhizopus delemer* (Synonym *R. oryzae*) in

UniProt database with accession number P0C1H7. The 4 matched peptides observed in mass spectra are shown in Fig 1c, which together accounted for 21% sequence coverage. The amino acid sequence of P0C1H7 was searched against the *R. oryzae* genome database using tBLASTn program which identified its corresponding 495 bp long ORF. The sequence information of the allergen was deposited in NCBI under the accession number KT734861. The allergen was assigned with the official designation as Rhi o 2 by the allergen nomenclature subcommittee of International Union of Immunological Societies (IUIS). The full-length Rhi o 2 cDNA was amplified by gene-specific PCR and was then cloned into pET15b+ vector for expression in *E. coli* with N-terminal 6xHis tag. Upon IPTG induction, the recombinant Rhi o 2 (rRhi o 2) was found to be present in the soluble fraction of the cell lysates and approximately 10 mg of rRhi o 2 could be purified from 1 L culture by Ni-NTA affinity chromatography (Fig 2a). The purified rRhi o 2 reacted with IgE-antibody in western blots (Fig 2b) with sera from 8 patients allergic to *R. oryzae* and 2 atopic asthma patients sensitized to *Aspergillus fumigatus*.

Rhi o 2 induced basophil activation – In addition to IgE-reactivity, the allergenic activity of rRhi o 2 was tested by its ability to stimulate mediator release from a rat basophil leukemic (RBL) cell line expressing the human high affinity receptor for IgE as shown in Fig 3. In this assay, the RBL-2H3 cell line expressing the receptor of human IgE-antibody (FcεRI) was first passively sensitized with IgE-antibodies from patient serum followed by challenge with increasing concentrations of purified rRhi o 2 that resulted in degranulation. The release of β-hexosaminidase started at 10 ng/ml concentration of the allergen, and the highest percentage of release took place at a concentration of 100 ng/ml. At this concentration, the percentage of release was found within a range from 26% to 48% among the 12 sera tested. After further increasing the rRhi o 2 concentrations, a decrease in degranulation was observed as evident from the typical bell-shaped dose-response curve. In addition to 10 Rhi o 2-reactive sera, 2 *Aspergillus*-positive sera (E1 and E2) were tested where rRhi o 2 was also able to induce

degranulation. In the control experiments, the percentage of release from RBL cells was below 5% as shown in supplementary Fig S1.

rRhi o 2 is a folded and monomeric cyclophilin–

The folding pattern of rRhi o 2 was determined by analyzing a far-UV circular dichroism (CD) spectrum (Fig 4a), which upon deconvolution in CAPITO software indicated a folded protein with 39% α-helices, 37% β-sheets, and 24% of turns/coils. The minimum was obtained 223 nm along with a shoulder around 213 nm and a characteristic rise towards the maximum below 205 nm. The thermal denaturation and refolding patterns of rRhi o 2 were studied by a step-scan procedure in which the CD spectra were taken to record the molar ellipticity [θ] signals at 223 nm during an ascending scan (from 20 to 90 °C) as shown in the melting curve of Fig. 4b. Transition temperature (T_m) was observed at 62°C at which the protein unfolding took place. At 90°C, rRhi o 2 displayed a sigmoidal unfolding transition to a complete random coil conformation. After cooling down the system (descending scan), a noticeable increase in the molar ellipticity signal at 223 nm was observed indicating a partial refolding of rRhi o 2 to regain 61% of its native conformation upon cooling. The aggregation status of the purified rRhi o 2 was studied by employing size exclusion chromatography. In the chromatogram shown in Fig. 4c, it was observed that under physiological condition, purified rRhi o 2 was eluted as a monomer of desired molecular weight. It was reconfirmed in non-reducing SDS-PAGE, where the purified rRhi o 2 protein migrated as a single band at a region corresponding to its theoretical molecular mass. The functional activity of rRhi o 2 was determined by rotamase enzyme assay, as shown in Fig. 4d. It was observed that the presence of rRhi o 2 caused 1.75 folds increases in the cleavage rate of the substrate peptide (N-Suc-Ala-Ala-*cis*-Pro-Phe-*p*-nitroanilide) by a proteolytic enzyme (chymotrypsin) as compared to the enzyme-blank (uncatalyzed) control set, which showed thermal isomerization of the substrate peptide. The higher rate of proteolytic cleavage in the presence of rRhi o 2 reflected an accelerated *cis* to *trans* isomerization of the Ala-Pro peptide bond due to PPIase activity. The results confirmed

that the recombinant allergen is an enzymatically active cyclophilin.

rRhi o 2 shows a broad range of cross-reactivity with other cyclophilin allergens – The IgE-cross reactivity of purified Rhi o 2 with 8 different cyclophilins reported from various allergy-eliciting sources was studied by competitive IgE-inhibition assays. In IgE-immunoblot inhibition (Fig. 5a), the overnight pre-incubation of patient serum pool with 5 µg/ml of rRhi o 2 resulted in a substantial inhibition of IgE-reactivity to each of the 4 recombinant cyclophilins (Asp f 11, Asp f 27, Mala s 6, and human CypA) on PVDF membrane as compared to the uninhibited control without pre-incubation. Complete IgE-inhibitions were observed when the inhibitor concentration was doubled. The result of immunoblot inhibition was in agreement with the results of ELISA inhibition assay (Fig 5b) in which each of the 4 recombinant cyclophilins in fluid phase inhibited binding to the plate-bound rRhi o 2 up to different extents. Maximum (71%) and minimum (48%) inhibition were observed for rMala s 6 and human CypA respectively at their highest concentration (i.e., 10 mg/ml) in the reaction mix. The normalized IC₅₀ values (minimum concentration required for 50% inhibition of IgE-antibody binding to the immobilized antigen) were calculated to be 1.587 µg/ml for rMala s 6, 2.032 µg/ml for rAsp f 11, 2.581 µg/ml for rAsp f 27, and 5.887 µg/ml for human CypA. In addition to these 4 recombinant cyclophilins, we also selected the crude antigenic extracts of house dust mite, carrot, tomato, and Indian birch pollen which have been reported to contain allergenic cyclophilins. As illustrated in Fig 5c, the addition of rRhi o 2 to patient sera containing specific IgE against cyclophilin led to the partial inhibition of IgE-binding to the ~18 kDa allergen present in the corresponding extracts.

Mapping the IgE-epitope of Rhi o 2 by amino acid deletion – In this study, we attempted to identify the IgE-binding region of cyclophilin through strategic mutagenesis of the Rhi o 2 primary structure. Total five different deletion mutants of Rhi o 2 were generated as schematically illustrated in Fig 6a. Four truncated versions of Rhi o 2 (TV-1 to TV-4) were created

by stepwise removing 10 amino acids from each of the N- and C-terminal sides and a fifth truncated version was generated by removing a conserved internal stretch of 69 amino acids (ΔRhi o 2). The molecular weights of all the 5 truncated Rhi o 2 variants were verified in 15% reducing SDS-PAGE as shown in Fig 6b-c. First, the IgE-reactivity of TV-1 to TV-4 was checked by immuno-dot blot with sera from cyclophilin allergic patients. As shown in Fig 6d, the IgE-binding was retained by Rhi o 2 derived TV-3 [Q³¹-E¹³⁴] after removal of up to 30 amino acids from each of either terminus of the allergen. Further removal of terminal amino acids from Rhi o 2 resulted in a complete loss of IgE-antibody binding as observed in TV-4 [T⁴¹-G¹²⁴]. Hence, in this strategy of terminal truncation, TV-3 can be considered as the minimal fragment of Rhi o 2 responsible for IgE-binding. However, none of these 3 IgE-reactive fragments (TV-1 to TV-3) was able to induce basophil degranulation (Supplementary Fig S2). Hence, TV-3 fragment was not considered as having an intact IgE-epitope and rather its IgE-reactivity was thought to be mediated by the presence of the remaining fractions of IgE-epitope left after truncation. Hence, sequence-based *in silico* analyses followed by experimental validations were performed to identify the intact IgE-epitope of Rhi o 2. A multiple sequence alignment (Supplementary Fig S3) of Rhi o 2 with six other allergenic cyclophilins revealed the presence of a highly conserved stretch of 69 amino acids (N⁸¹-N¹⁴⁹ fragment), which was found to have a high degree of antigenic propensity values and solvent accessibility suggesting the possible presence of immunodominant B cell epitopes as analyzed in DNASTAR Lasergene version 7.2. (DNASTAR Inc., Madison, WI, USA). The C-terminus of TV-3 was 15 residues shorter than that of the N⁸¹-N¹⁴⁹ fragment. This additional stretch of 15 residues was not only conserved among cyclophilins but also highly surface exposed as shown in Supplementary Fig S4. Hence, we anticipated the N⁸¹-N¹⁴⁹ fragment as the possible site of Rhi o 2, where a functional IgE-epitope was formed. This was confirmed by generating a fifth mutant ΔRhi o 2 with deleted N⁸¹-N¹⁴⁹ fragment. The ΔRhi o 2 was found to have no reactivity with IgE-antibodies from any of the 10 patients' sera in the

non-denaturing RAST-based immuno-dot blot shown in Fig 6e. Moreover, the polyclonal rabbit IgG antibodies raised against Δ Rhi o 2 were unable to inhibit IgE-binding (~22.3%) to rRhi o 2 in a competitive ELISA inhibition assay shown in Fig 7. In contrast, anti-TV-3 rabbit IgG was able to inhibit ~79.4% of patients' IgE-binding to plate-bound rRhi o 2. Highest level of IgE-inhibition (~86.3%) was observed with rabbit IgG raised against the intact Rhi o 2 in which the N⁸¹-N¹⁴⁹ stretch remained intact. A similar pattern of IgE inhibition was observed when these IgG antibodies in rabbit antisera were used against 4 other recombinant cyclophilins in parallel experiments. The findings demonstrated that the conserved N⁸¹-N¹⁴⁹ fragment of Rhi o 2 represented the antigenically active molecular domains responsible for IgE-recognition. Hence, we selected N⁸¹-N¹⁴⁹ fragment for the next epitope grafting experiment.

Increasing IgE reactivity and allergenic activity of yeast cyclophilin by replacing the conserved 69 amino acid residue of Rhi o 2 – Yeast cyclophilin CPR1 (UniProt: P14832) is considered as a non-allergenic member of cyclophilin family. Despite having 67.9% and 72.84% sequence identities with Rhi o 2 and Mala s 6 respectively, CPR1 was never reported to show IgE-mediated allergic inflammation in atopic individuals. Here, we selected CPR1 as a scaffold in which the conserved N⁸¹-N¹⁴⁹ fragment of Rhi o 2 was grafted by replacing the native homologous region of CPR1 protein as illustrated in Fig 8a. This epitope grafting resulted in the creation of a hybrid cyclophilin (Hybrid_Cyp) which showed a structural fold similar to Rhi o 2 (RMSD 0.368). On the other hand, the alignment of Rhi o 2 homology model with the crystal structure of CPR1 (PDB: 1IST) resulted in an RMSD value 0.425 (Fig 8b). Recombinant CPR1 and hybrid cyclophilin were cloned, expressed in *E. coli* and purified, as shown in Fig 8c for subsequent analysis. CD spectra of the Hybrid_Cyp showed a properly folded protein with secondary structural elements similar to that of the Rhi o 2 than to CPR1 (Fig 9a), which was also evident from the structural alignment analysis. In ELISA inhibition assay shown in Fig 9b, Hybrid_Cyp displayed 12 folds higher IgE-binding than CPR1 (inhibition

percentage of Hybrid_Cyp at IC₅₀; i.e. 50%: inhibition percentage of CPR1 at the same concentration; i.e. 4.2%). Hybrid_Cyp at its highest concentration of 10 mg/ml as a fluid phase inhibitor resulted in 78% IgE-binding inhibition to plate-bound WT rRhi o 2 (IC₅₀; 0.1501 µg/ml). In addition to IgE-reactivity, Hybrid_Cyp was found to gain allergenic activity after epitope grafting. As shown in Fig 9c, 100 ng/ml of Hybrid_Cyp was able to induce degranulation from IgE-sensitized basophils at a level comparable to that of the WT rRhi o 2. CPR1 could not release significant level (<5%) of β -hexosaminidase, and it was 18 folds (ratio between the mean release by Hybrid_Cyp and CPR1 at 100 ng/ml) lower than that of the Hybrid_Cyp.

DISCUSSION

Patients sensitized to cyclophilins, a family of highly conserved chaperones, may show allergic reactions to fungi (12), pollens (19, 20), mites (21), and foods (22, 23) of diverse taxonomic origins. In the present study, we report a new cross-reactive cyclophilin allergen Rhi o 2, which showed IgE-reactivity within a mold-allergic population suffering from broad cross-reactivity. Based on an earlier immunoproteomic report on *R. oryzae* allergens (24), the present study was initiated with purifying the Rhi o 2 allergens in natural form and then as recombinant form. rRhi o 2 displayed IgE-reactivity and allergenic activity with the patient sera tested. rRhi o 2 was obtained in properly folded monomeric form displaying functional PPIase activity characteristics of most of the reported cyclophilins except Asp f 11, which forms dimer by 3D domain swapping (15). Therefore, Rhi o 2 can be considered as a marker allergen for molecular diagnosis of mold allergy prevalent in India and other tropical countries (24-27). The percentage of sequence similarity of Rhi o 2 with 4 other cyclophilins was in agreement with the respective degree of cross-reactivity between Rhi o 2 and these allergens. Mala s 6 (69%) and human CypA (52%) displayed the maximum and minimum IgE-inhibition respectively against the immobilized Rhi o 2. Presence of the similar antigenic determinants commonly shared by Rhi o 2 and its human homolog CypA increase the chances of allergen-

homologous autoantigen mediated perpetuation of atopic diseases in sensitized individuals (28). In IgE-inhibitions with allergen extracts, weak IgE-binding signals were still visible in inhibited blots. This can be explained as either the presence of cyclophilins in these extracts that are more potent than Rhi o 2 or the presence of another allergen of similar MW. Further detailed study is warranted to determine the original source of sensitization among the cross-reactive cyclophilins in this group of patients. Some previous reports exist on the putative IgE-epitopes of cross-reactive cyclophilins (20, 29). However, those findings were not experimentally validated. Another study on Bet v 7 suggested the 7 residue loop specific for plant cyclophilins as the possible mediator of IgE-cross reactivity (19). However, a later study on periwinkle pollen allergen Cat r 1 ruled out this hypothesis by generating a loop-deletion mutant that was still capable of IgE-binding (20). In the present study, a protein engineering approach was undertaken to identify the conserved IgE-binding region of Rhi o 2 responsible for cross-reactivity. Sequential 10 residues truncation on either side of Rhi o 2 identified TV-3 as the smallest polypeptide unit of the allergen capable of IgE-binding. However, TV-3 represented partial but not the entire IgE-epitope as TV-3 was unable to induce degranulation. The sequence analyses of cyclophilins revealed substantial degree of sequence conservation throughout the length of the protein. But we focused on the N⁸¹-N¹⁴⁹ fragment as the possible antigenic region of Rhi o 2 due to its high level of surface exposure and antigenic propensities. Certain earlier reports (20, 29) also suggested the possibility of putative IgE-epitopes in this region. N⁸¹-N¹⁴⁹ fragment was found to have an additional stretch of 15 solvent accessible residues at its C-terminal end and the rest of its sequence overlapped with the C-terminal part of TV-3. It was observed that the interference in IgE-binding by polyclonal IgG against Rhi o 2 containing the entire N⁸¹-N¹⁴⁹ fragment was higher than that of the TV-3. Moreover, the IgG against ΔRhi o 2 containing a complete deletion of N⁸¹-N¹⁴⁹ fragment did not significantly hamper IgE-binding to Rhi o 2. Taken together, these observations suggested that the IgE-epitope of Rhi o 2 had more overlaps with N⁸¹-N¹⁴⁹ fragment than with TV-3. Hence, N⁸¹-N¹⁴⁹ fragment was selected

for epitope grafting experiment. Epitope grafting seemed to be a powerful tool as it previously helped to identify the crucial residues responsible for cross-reactivity between Bet v 1 (birch pollen) and Mal d 1 (apple fruit) leading to OAS (30). In another similar study, grafting of the IgE-specific N-linked glycosylation from plant glycoallergen into non-allergenic horse heart myoglobin conferred IgE-reactivity to the non-allergen (31). In line with the findings of these previous reports, here we took an attempt to verify the possibility of N⁸¹-N¹⁴⁹ fragment as the antigenic determinant of Rhi o 2 by epitope grafting approach. Yeast cyclophilin CPR1, a close homolog of Rhi o 2 and Mala s 6, was previously found to react with IgE-antibodies from cyclophilin positive sera (12); however, no clinical pieces of evidence exist on the allergenic activity of CPR1. Here, the grafting of the antigenic N⁸¹-N¹⁴⁹ fragment by replacing the homologous region from CPR1 primary structure was found to convert CPR1 into a degranulation-inducing protein with enhanced IgE-binding. The results are in agreement with the presence of higher structural similarity of Hybrid_Cyp with Rhi o 2 than that with CPR1. Hence, it can be ascertained that N⁸¹-N¹⁴⁹ fragment is the primary site of IgE-epitope formation, which is thought to be directed by the overall conformational folds of the protein. The conformational nature of IgE-epitope of Rhi o 2 is supported by two observations emerged from the present study. Firstly, the Rhi o 2-derived TV-1 to TV-3 mutants, despite having IgE-reactivity, didn't induce basophil degranulation suggesting the truncation induced conformational changes in the epitope structure. Secondly, the TV-4 mutant after the fourth round of truncation was found to abolish IgE-binding capacity, albeit it retained 70% of amino acids of the N⁸¹-N¹⁴⁹ fragment. The refolding behavior of Rhi o 2 as observed in its melting curve also corroborates the possibility of conformational epitope mediated IgE-binding in immunoblot under denaturing conditions. Taken together, the present study not only reports a comprehensive characterization of the new fungal cyclophilin Rhi o 2 as a diagnostic antigen for mold allergy but also generates structural information on cyclophilin cross-reactivity. It is therefore tempting to speculate that this cross-reactive IgE-epitope information can be rationally

utilized for a future paradigmatic approach to construct genetically engineered immunotherapeutics against atopic diseases caused by a broad range of cyclophilin allergens from various sources.

EXPERIMENTAL PROCEDURES

Human Subjects – The residual sera were obtained in a reciprocated and anonymous manner from 10 fungal allergic patients (P1 to P10) with respiratory symptoms visiting the OPD of B. R. Singh Hospital and Center for Medical Education and Research, Kolkata, India. These patients were selected on the basis of their clinical history of allergy and seropositive IgE-level against *R. oryzae* antigen. Sera of 2 atopic asthma patients sensitized to *A. fumigatus* (E1 and E2), and non-atopic sera from 2 healthy volunteers (N1 and N2) were also collected. The entire study was approved by the Bose Institute human ethics committee (Reference no. BIHEC/2014-15/4) following the declaration of Helsinki principles and informed written consents were taken from the participants. Patients with chronic parasitic infections were excluded. Clinical and demographic details of the participants are given in Supplementary Table S1.

Column chromatography – About 20 gm of the lyophilized spore-mycelial mat of *R. oryzae* was homogenized in 20 mM sodium phosphate buffer (pH 7.8) containing 5% glycerol, 1 mM EDTA and 0.5 mM PMSF (buffer A). The lysate was saturated with 50% ammonium sulfate followed by resuspension of the pellet fraction in buffer A. The resuspended fraction was then incubated with DEAE Sephacel gel (Sigma-Aldrich, USA) equilibrated in the same buffer and the resulting supernatant was loaded onto HiTrapTM CM FF column (GE Healthcare, Sweden). The column bound proteins were eluted in 0.5 ml fraction with 0–1 M NaCl gradient at a flow rate of 0.2 ml/min. Fractions were checked in reducing SDS-PAGE for the presence of the desired allergen.

IgE western blot and Immuno dot blot – Immunoblots were performed with 1:10 v/v diluted patient sera as already described in (32). Bound IgEs were detected either with monoclonal anti-human IgE with AP conjugate (Sigma, USA)

and NBT BCIP (Abcam) or with ¹²⁵I labeled anti-human IgE 1:10 v/v followed by autoradiogram (33).

LC-MS/MS – The desired protein band was gel excised and trypsin digested (34). Peptides were fractionated in 1.8 µm CSH C18 300 µm X 100 mm NanoEase analytical column fitted with ACCQUITY UPLC M-Class and then subjected to Xevo® G2-XS QToF mass spectrometry (Waters Corporation, Milford MA, USA) in ESI (+ve) platform at 2.5 kV (35). Raw MS/MS spectra were processed and analyzed in Progenesis QI search engine (Waters Corporation, Milford MA, USA) against the UniProt database using the standard search parameters.

cDNA cloning and recombinant proteins – The full-length Rhi o 2 cDNA was PCR amplified from the first-strand cDNA synthesized from the TRIzolTM (Invitrogen, Germany) extracted RNA of *R. oryzae* using iScriptTM kit (Bio-Rad, USA). The cDNA was cloned into pET15b vector (Novagen, EMD Millipore, USA) with N-terminal hexahistidine tag (36). After sequence confirmation, the recombinant protein was expressed in *E. coli* BL21(DE3)Rosetta cells with 0.5 mM 1-thio-β-d-galactopyranoside for 12 h at 20°C. The Rhi o 2 protein was purified in native form using Ni-NTA beads (Qiagen, Germany) as already described (37). rMala s 6 was expressed in *E. coli* and purified as described in (38). cDNA cloning, bacterial expression, and recombinant protein purification strategies of the other 11 cyclophilin constructs used in this study are illustrated in Table 1. Full-length ORFs of Asp f 11, Asp f 27, and yeast CPR1 were amplified from the first-strand cDNA preparations of the corresponding mold species. Yeast first-strand cDNA preparation was a kind gift from Dr. Nandini Ghosh, Vidyasagar University, Midnapore, India. Codon-optimized synthetic G-blocks were purchased from IDT Inc., IA, USA. Recombinant proteins (TV-3 and TV-4) expressed in inclusion bodies were purified under denaturing condition using 7M urea buffer and then made soluble by rapid dilution method as described in (39).

Allergen extracts – Glycerinated aqueous (1:20 w/v) extracts of dust mite (*Dermatophagoides*

farinae), birch, tomato, and carrot were purchased from Creative Diagnostic Medicare Private Limited, Mumbai, India. The birch pollen extract was purchased from HollisterStier Allergy, WA, USA. The extracts were concentrated by UPPA™-I & II Pack (G-Biosciences®, USA) to achieve a protein concentration 5 - 7 mg/ml and dissolved in SDS-loading buffer.

Basophil activation test (BAT) – BAT was performed with RBL-2H3 cell line (ATCC CRL-2256) using 1:10 v/v diluted sera and increasing concentrations (1-1000 ng/ml) of rRhi o 2. The percentage of release was calculated as described (40).

Gel filtration – About 1 mg of rRhi o 2 was run through Superdex 75 Increase 10/300 GL (GE Healthcare, Sweden) column as described (41) with 0.5 mg of bovine cytochrome c as standard.

CD spectroscopy – About 4.8–5.0 µM of the dialyzed purified protein was buffer exchanged with 5 mM NaH₂PO₄ (pH 7.8) and 1% glycerol. CD spectra were recorded as described in (42). The raw data in millidegree were converted into molar ellipticity, and the folding patterns of the proteins were analyzed in CAPITO server (43).

Rotamase assay – As described in (44), 1 ml of reaction mixture containing 20 nMrRhi o 2, and 80 µM N-succinyl-ala-ala-cis-pro-p-nitroanilidine in assay buffer (50 mM HEPES, pH 8; 150 mM NaCl; and 0.05% Triton-X) was incubated for 360 seconds at 15°C. Cleavage of the *trans* isomer of the chromogenic substrate peptide by 300 µg/ml chymotrypsin was monitored by recording the absorbance at 390 nm in a Peltier controlled UV-250 spectrophotometer (Shimadzu, Japan). A control reaction mixture was prepared without rRhi o 2 to record the spontaneous thermal isomerization of the peptide.

Immunoblot inhibition and ELISA inhibition – About 5 µg/lane of each of the 5 recombinant cyclophilins or 100 µg/lane of each of the 4 allergen extracts was transferred onto PVDF membrane. The membranes were then exposed to

either a pool of 3 sera (P2, P5, and P6) with high titer (4 folds higher than non-atopic sera) of specific IgE against Rhi o 2 (uninhibited) or a serum pool pre-incubated separately with 5 and 10 µg/ml of rRhi o 2 (inhibited) for overnight. For ELISA inhibition, plate-bound (1 ng/µl) rRhi o 2 was exposed to sera pool preincubated with gradually increasing concentrations (0.001-10000 µg/ml) of various cyclophilins. Bound IgEs were detected with anti-human IgE-AP 1:1000 v/v (Sigma, USA, A3076) and pNPP (Abcam). The percentage of IgE-inhibition was calculated as described earlier (40).

Rabbit antisera and competitive ELISA – Polyclonal antisera against rRhi o 2, TV-3 and ΔRhi o 2 were generated in New Zealand white rabbit by Biobharati Lifesciences Pvt. Ltd., Kolkata, India. For competitive ELISA with rabbit IgG, polystyrene plates were coated with 5 ng/µl of recombinant cyclophilins and incubated with either preimmune sera or antisera at 1:100 v/v dilutions for 8 h at room temperature. Plates were then washed 5 times and then exposed to 1:10 v/v diluted patient sera (n = 8) for overnight at 4°C. Bound IgEs were detected and the percentage of IgG mediated IgE-inhibition was calculated for each patient using the following formula –

$$\% \text{ of IgE inhibition} = \left[1 - \frac{A_{405} \text{ of antisera (with specific IgG)}}{A_{405} \text{ of preimmune sera (without specific IgG)}} \right] \times 100$$

Bioinformatics study – The energy minimized homology models of Rhi o 2 and Hybrid Cyclophilin were built using SwissModel server (45) as described earlier (36). The 3D models were then aligned with the crystal structure of yeast CPR1 using PyMol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) to calculate the RMSD. Sequence alignments were performed in Clustal Omega online tool (46).

Statistical analyses – GraphPad Prism v8 was used to calculate (i) the significance of difference between IgE inhibition by anti-Rhi o 2 versus anti-TV-3 (p<0.05) and versus anti-ΔRhi o 2 (p<0.001) IgG using paired Students' t test and (ii) the IC₅₀ values of the cross-reactive allergens using nonlinear regression analysis.

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FOOTNOTES

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Abbreviations used are: β ME, beta mercaptoethanol; MW, molecular weight; kDa, kilodalton; WHO/IUIS, World Health Organization/International Union of Immunological Societies; HSA, Human Serum Albumin; BSA, Bovine Serum Albumin; CD, Circular dichroism.

Table 1 – Cloning, bacterial overexpression, and purification strategies of the various cyclophilin constructs used in the present study. Full-length cDNA was obtained either by PCR amplification from first-strand cDNA preparation (FSCP) or by Gene synthesis (GS) or by deletion mutagenesis on Rhi o 2 construct (DMRC). Recombinant protein was expressed either in native soluble (S) form or in inclusion bodies (IB).

Cyclophilin constructs	cDNA from	Vector	6xHis tag	Expression parameters				Protein state
				<i>E. coli</i> strain	IPTG (mM)	Time (h)	Temp. (°C)	
Asp f 11	FSCP	pET21d	N-	BL21(DE3)	0.5	12	20	S
Asp f 27	FSCP	pET22b	N-	BL21(DE3)	0.5	12	16	S
Hum. CypA	GS	pET28a	N-	BL21(DE3)	1.0	3	37	S
TV-1	DMRC	pET15b	N-	Rosetta	0.5	12	20	S
TV-2	DMRC	pET15b	N-	Rosetta	0.5	12	20	S
TV-3	DMRC	pET15b	N-	Rosetta	0.5	12	16	IB
TV-4	DMRC	pET15b	N-	Rosetta	0.5	12	16	IB
Δ Rhi o 2	DMRC	pET15b	N-	BL21(DE3)	0.5	12	20	S
CPR1	FSCP	pHisTEV	N-	Rosetta	1.0	3	37	S
Hybrid Cyp	GS	pHisTEV	N- and C-	BL21(DE3)	1.0	3	37	S

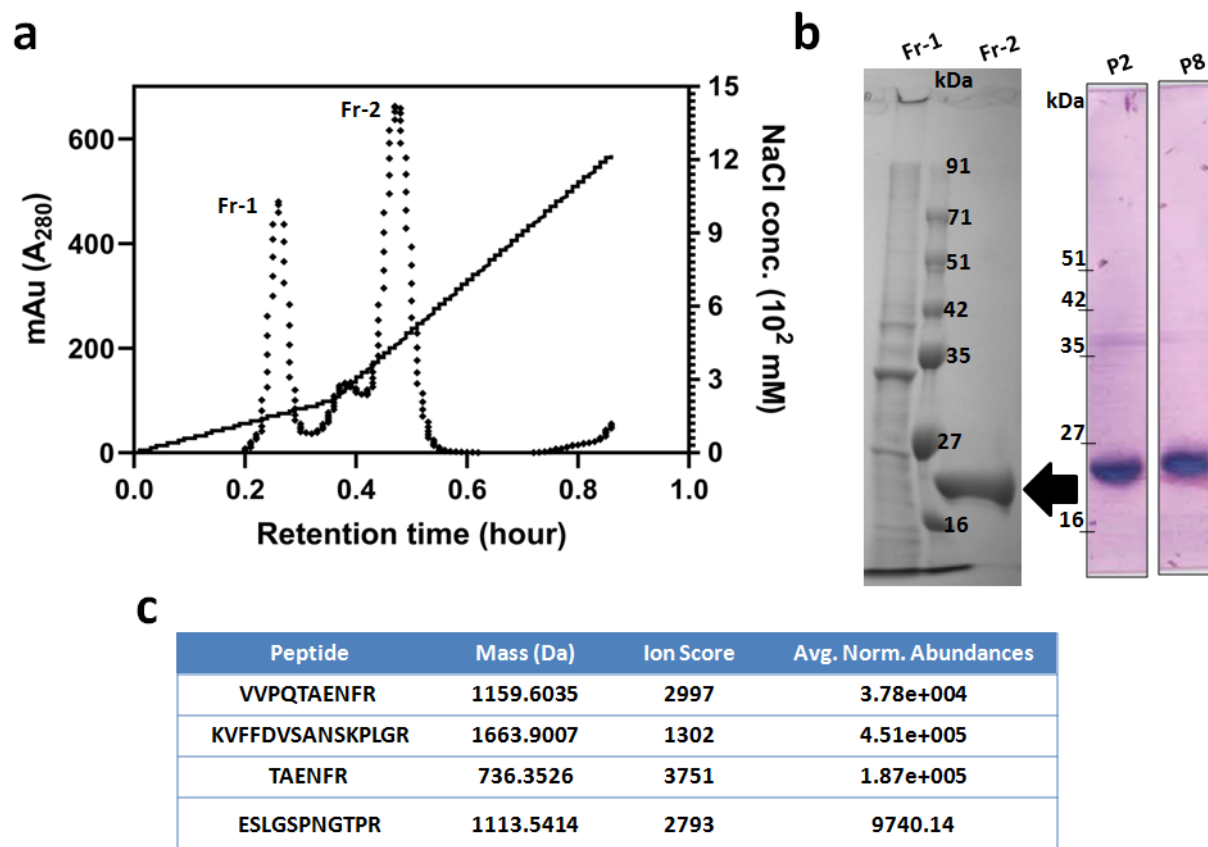


Fig 1: Purification and identification of natural nRhi o 2. (a) Chromatogram of fractions eluted from HiTrap™ CM FF column using NaCl gradient. Crude *R. oryzae* extract after ammonium sulfate cut was incubated with DEAE Sephacel gel and the supernatant was loaded onto Carboxymethyl cation exchange column. Absorbance of eluted fractions was read at 280 nm (y axis; left) as a function of time in hour (x axis) and using NaCl concentration gradient in mM (y axis; right). (b) The second fraction (lane Fr-2) was found to contain the desired 18 kD an Rhi o 2 in 12% SDS-PAGE (labeled with an arrow), which was immunoblotted with two patient sera (P2 and P8). (c) Results of LC-MS/MS analyses of nRhi o 2 showing the sequences of 4 matched peptides, their MW in Dalton, the peptide ion scores, and the average normalized abundance in the MS/MS spectra.

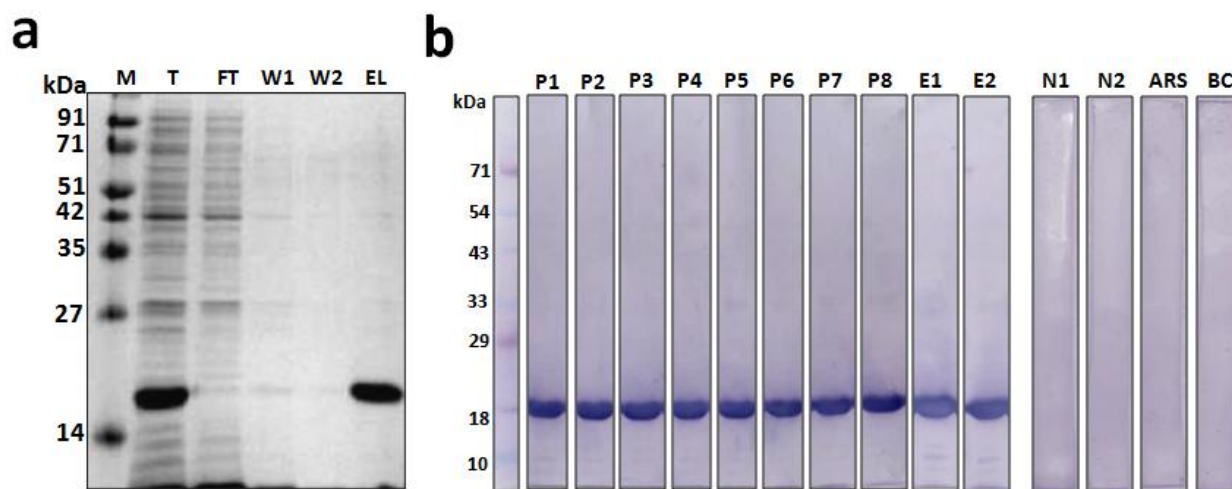


Fig 2: Recombinant rRhi o 2. (a) 12% SDS-PAGE showing Ni-NTA purification of 6xHis tagged rRhi o 2. After IPTG induction, the crude *E. coli* lysate (lane T) in lysis buffer containing 10 mM imidazole was loaded onto Ni-column and the flow through (lane FT) was collected. The Ni-column was then washed twice with wash buffer containing 40 mM imidazole (lanes W1 and W2) to remove non-specific *E. coli* proteins. Column bound rRhi o 2 was then eluted with 250 mM imidazole (lane EL). (b) IgE-reactivity of rRhi o 2 was tested by immunoblot using sera from 8 *R. oryzae*-sensitized patients (P1-P8) and 2 sera from *A. fumigates*-sensitized patients (E1, E2). Negative control blots using 2 sera from non-allergic subjects (N1, N2), 1 serum from an *Alternaria alternate*-reactive patient (ARS), and the buffer control (BC) without any serum.

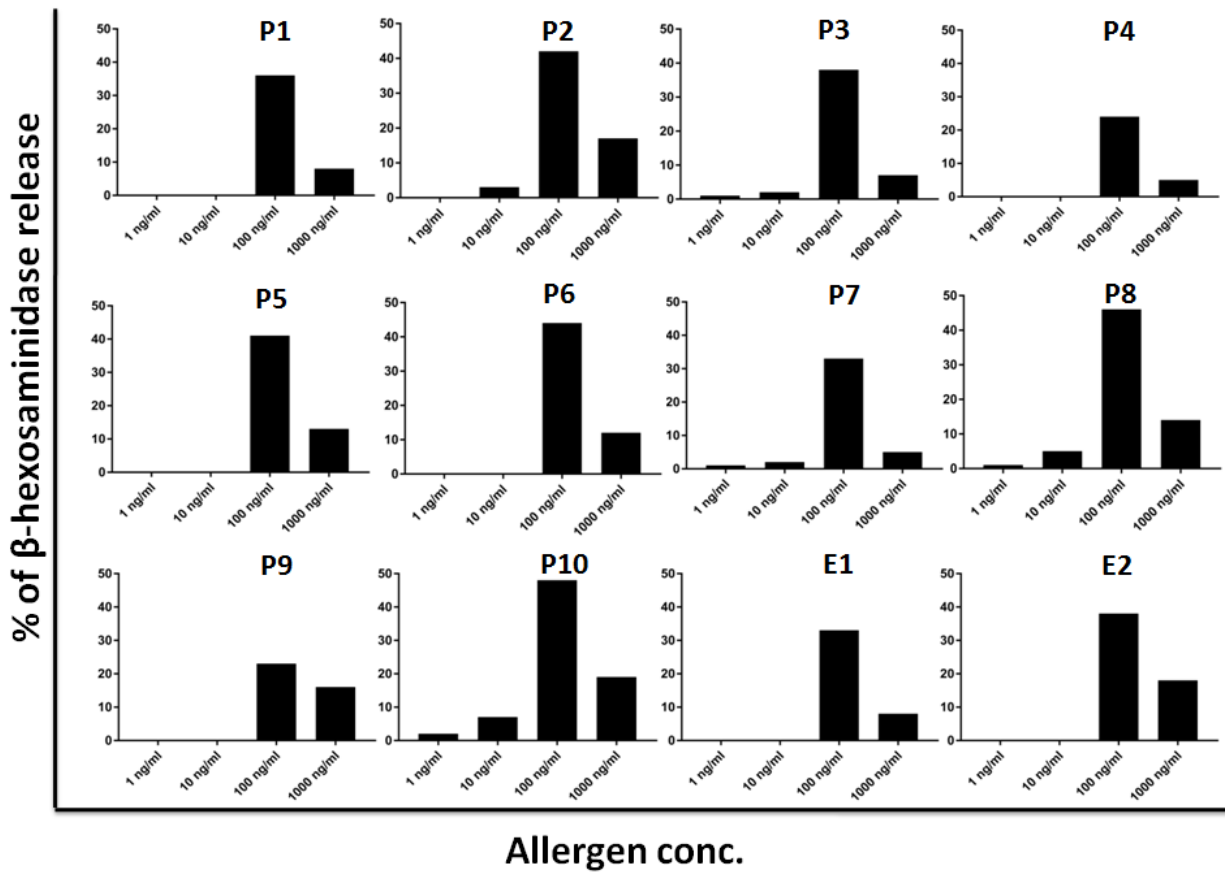


Fig 3: Allergenic activity of rRhi o 2: Increasing concentrations of rRhi o 2 (x axes) were used to stimulate the RBL cell line passively sensitized with IgE-antibodies from sera from 10 *R. oryzae* sensitized patients (P1-P10) and sera from 2 *A. fumigatus* allergic patients (E1, E2) resulted in a dose-dependent β -hexosaminidase release (y axes: percentages of total β -hexosaminidase release) from RBL cells.

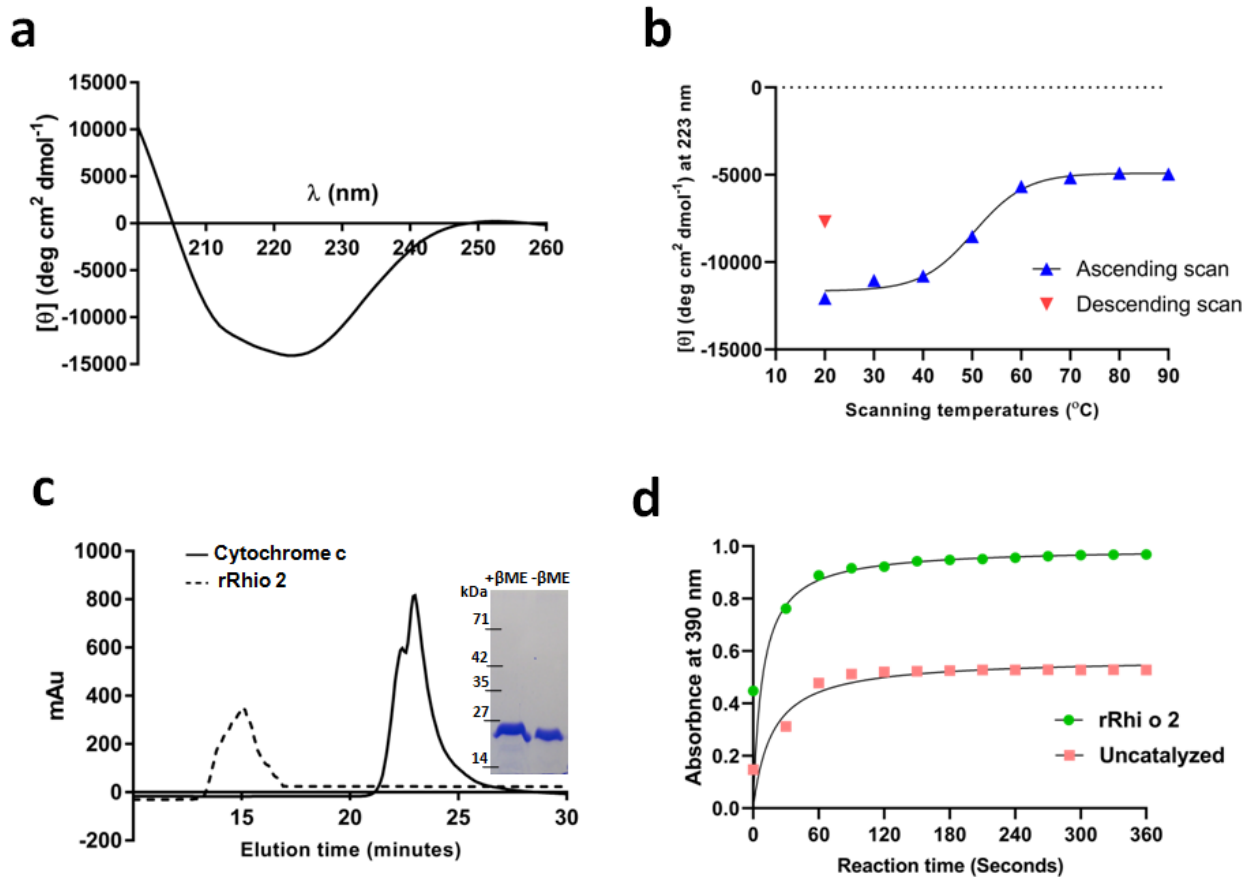


Fig 4: Biophysical and functional characterization of rRhi o 2: (a) CD spectra of 5 μM rRhi o 2 showing molar ellipticity (y axis) at various wavelength (x axis) at 25 $^{\circ}\text{C}$. (b) Melting curve of rRhi o 2 showing the refolding behavior of the allergen by recording molar ellipticities at 223 nm (λ_{max}) plotted in the y axis at various temperatures in x axis. With increasing temperature (Ascending scan) rRhi o 2 displayed a non-linear sigmoidal transition from folded to unfolded state. After cooling down (Descending scan), rRhi o 2 displayed partial refolding. (c) Chromatogram of rRhi o 2 eluted from Gel filtration column as a monomer. Absorbance of eluted fractions were read at 280 nm (milli Abs. unit at y axis) and plotted as a function of elution time from the column (x axis). Bovine cytochrome c (MW 12.3 kDa, was used as standard. No aggregation of rRhi o 2 was observed when run in 13% SDS-PAGE under reducing condition with βME versus non-reducing condition without βME . (d) PPIase activity of rRhi o 2 in rotamase assay showing increased *cis-trans* isomerization of ala-pro bond of the substrate peptide followed by chymotrypsin cleavage and concomitant increase in A_{390} (y axis) plotted as a function of time (x axis). Enzyme blank control showing spontaneous thermal isomerization (uncatalyzed).

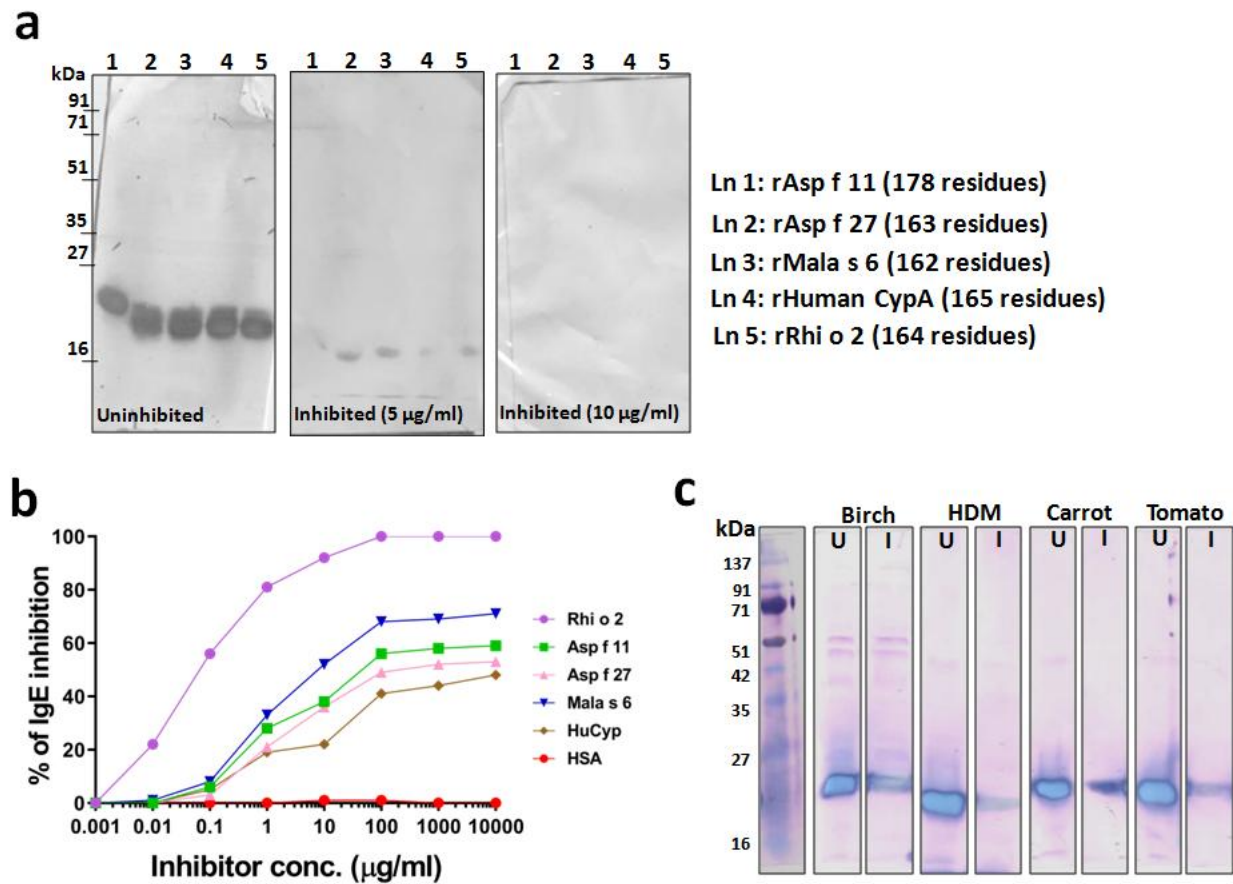


Fig 5: Cross-reactivity of rRhi o 2. (a) Immunoblot showing that 4 different purified cyclophilins (lanes 1-4; amino acid lengths are mentioned in right panel) reacted with IgE-antibodies from a pool of 3 Rhi o 2-allergic sera (1:10 v/v) and immunoblot inhibition when the serum pool was mixed separately with 5 µg/ml and 10 µg/ml of rRhi o 2 for overnight. rRhi o 2 on membrane (lane 5) used as positive control for auto-inhibition. (b) ELISA inhibition in which plate-bound rRhi o 2 was exposed to pooled sera (1:10 v/v) incubated with exponentially increasing concentration (x axis) of either 4 cyclophilins or rRhi o 2 (auto-inhibition) or HSA (negative control) or sera only (uninhibited). Cross-reactivity of each test antigen was plotted as the percentage (y axis) of inhibition of IgE-binding to immobilized rRhi o 2. (c) Immunoblot inhibition (I) showing inhibition of IgE-reactivity to cyclophilins present in crude extract of 4 allergenic sources when exposed to a pool of 3 Rhi o 2-allergic sera (1:5 v/v) mixed with 10 µg/ml rRhi o 2. In uninhibited blots (U), the proteins corresponding to inhibited bands showed IgE-reactivity with sera not mixed with rRhi o 2.

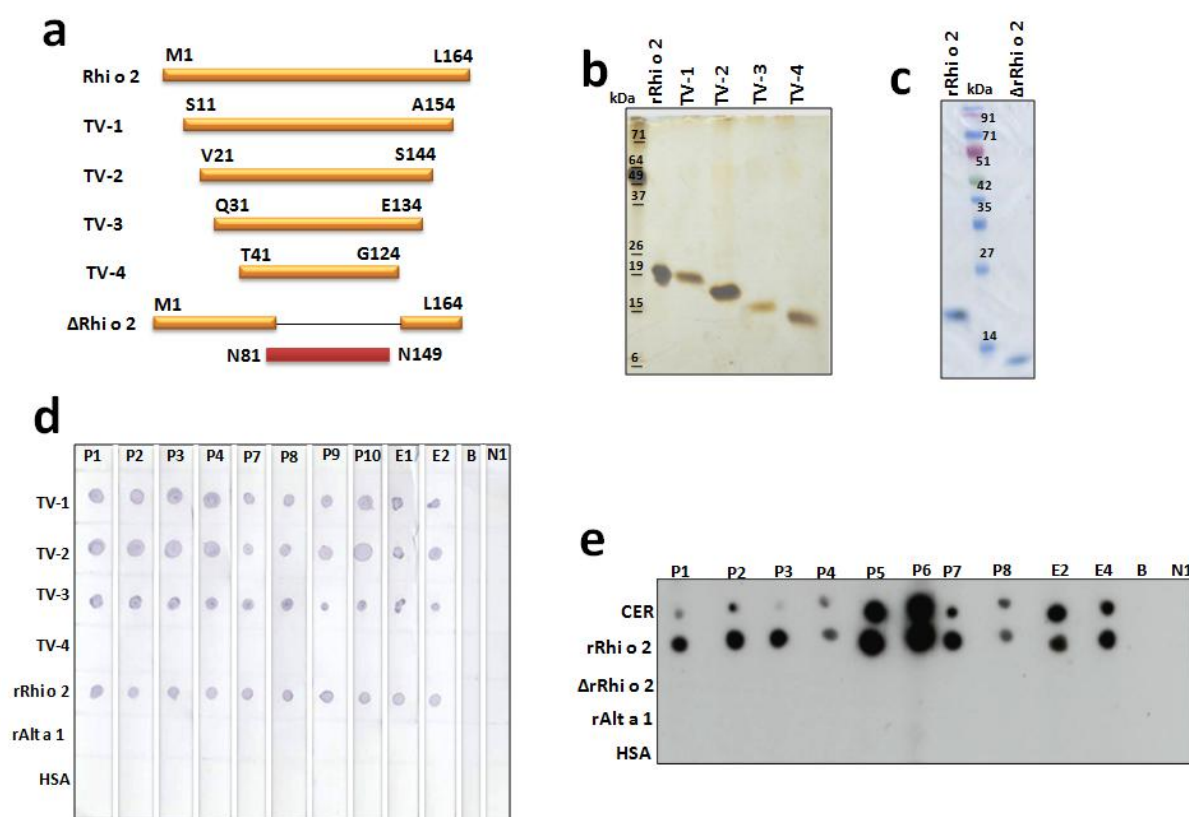


Fig 6: Mapping of Rhi o 2 IgE-epitopes. (a) Schematic representation of the design of 5 deletion constructs of Rhi o 2 and their 15% SDS-PAGE profile in (b) after silver staining and (c) after CBB-R250 staining. Following Ni-NTA purification, these proteins were purified by a second round of gel filtration to remove the impurities. (d) IgE-immunodot blot of 200 ng of each of the 4 truncated versions (TV-1 to TV-4) of Rhi o 2 or intact rRhi o 2 with either 10 patient sera (P1 to E2) or buffer control (B) or healthy sera (N1). (e) Autoradiogram of RAST based IgE-immunodot blot of 200 ng of each of either rRhi o 2 or ΔrRhi o 2 or cross-reactive rMala s 6 using 10 patient sera. For detection, ^{125}I labeled anti-human IgE was used. An unrelated allergen Alt a 1 and HSA were used as negative control antigens in the dot blots.

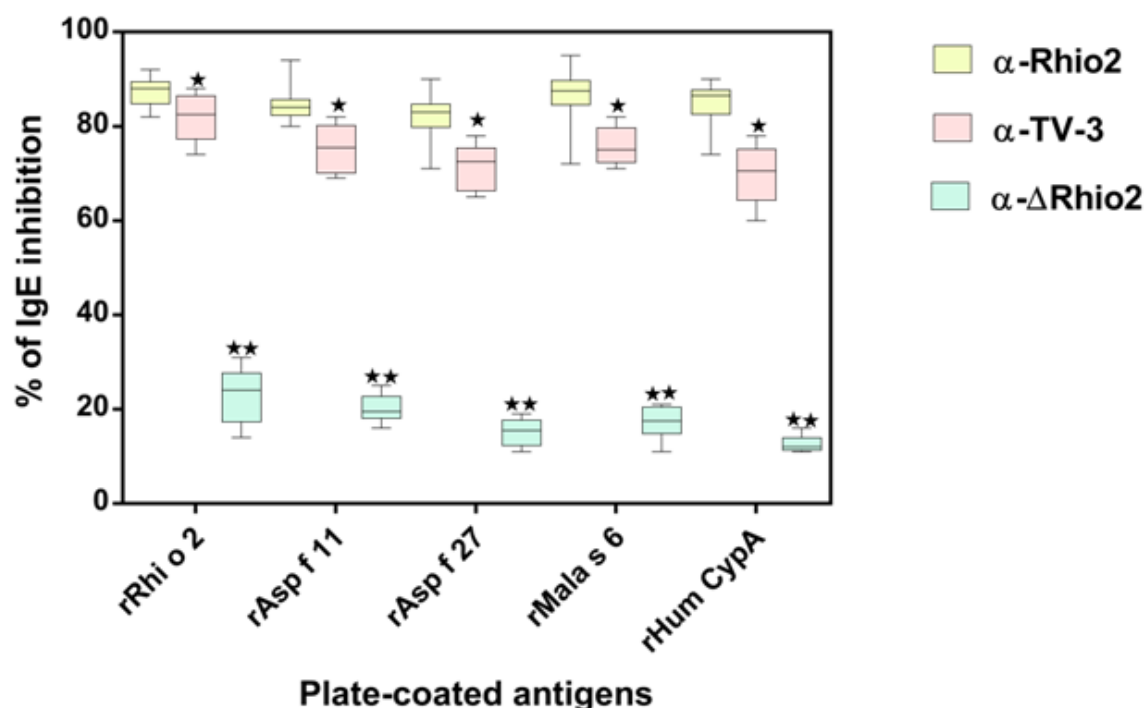


Fig 7: Inhibition of patient IgE by IgG in rabbit antisera. Competitive ELISA in which 5 different recombinant cyclophilin allergens were separately pre-incubated with IgG from either anti-Rhi o 2 or anti-TV-3 or anti-ΔRhi o 2 IgG from rabbit antisera (1:100 v/v) and then exposed to sera (1:10 v/v) from cyclophilin-allergic patients (n = 8). Corresponding preimmune rabbit sera were used for control purposes to calculate the percentage of IgE-binding inhibitions (y axis). Box-whisker plots with medians and minimum to maximum ranges. *, $p < 0.05$ for IgE-inhibition by anti-TV-3 versus anti-Rhi o 2 and **, $p < 0.001$ for IgE-inhibition by anti-ΔRhi o 2 versus anti-Rhi o 2 IgG, α- refers to anti-.

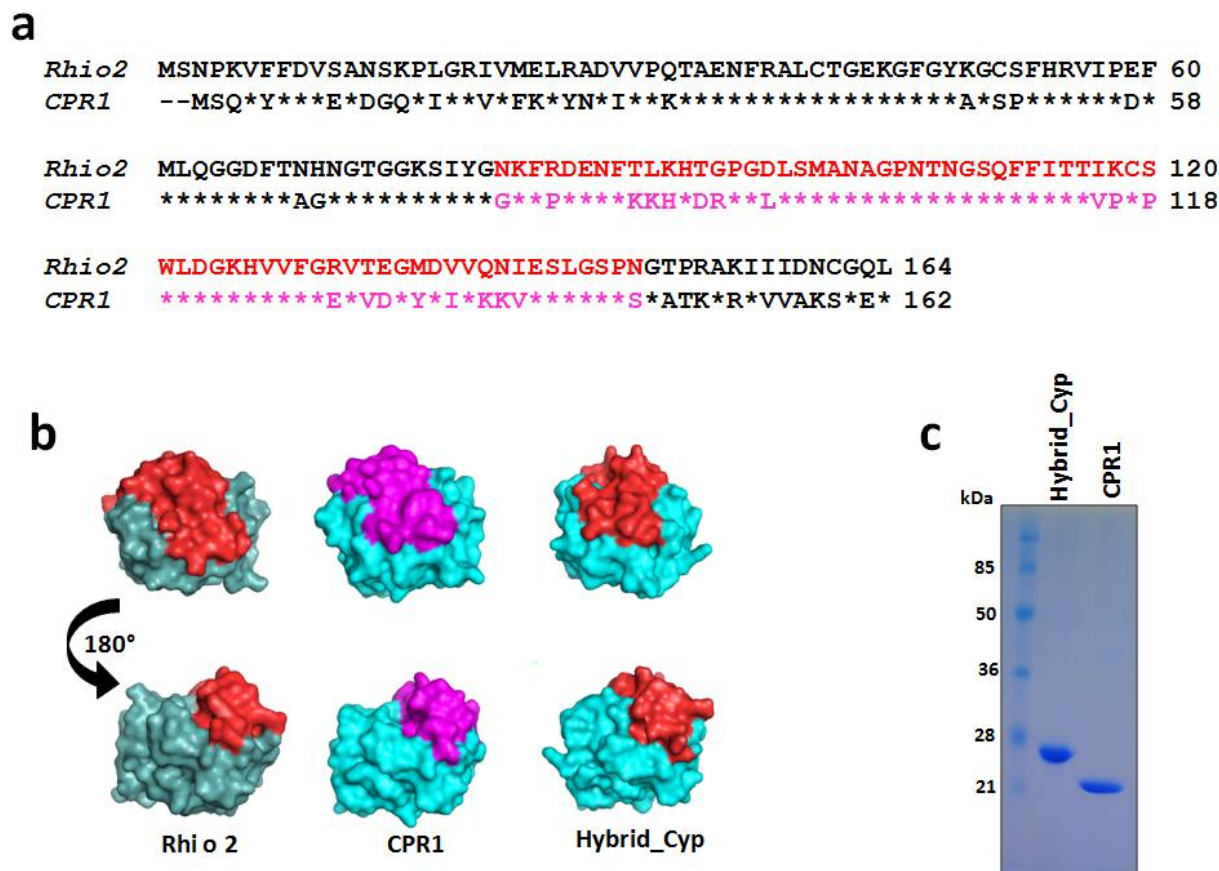


Fig 8: Epitope grafting. (a) Alignment of Rhi o 2 sequence with yeast CPR1 showing the identical residues as asterisks and gaps as dashes. N⁸¹-N¹⁴⁹ fragment on Rhi o 2 shown in red and its homologous stretch in CPR1 in pink. (b) Surface representation of front and back views (turned 180° on horizontal axes) of the homology model of Rhi o 2, crystal structure of yeast CPR1 (PDB: 1IST). A third energy-minimized homology model was built for the Rhi o 2:CPR1 hybrid cyclophilin (Hybrid_Cyp) in which N⁸¹-N¹⁴⁹ fragment of Rhi o 2 was grafted in CPR1 by replacing its homologous stretch G⁸²-S¹⁴⁷. (c) 15% SDS-PAGE profile of Hybrid_Cyp (N and C-terminal 6x His tags) and recombinant CPR1 (N-terminal 6x His tag) after Ni-NTA purification followed by gel filtration. MW maker is on the left margin.

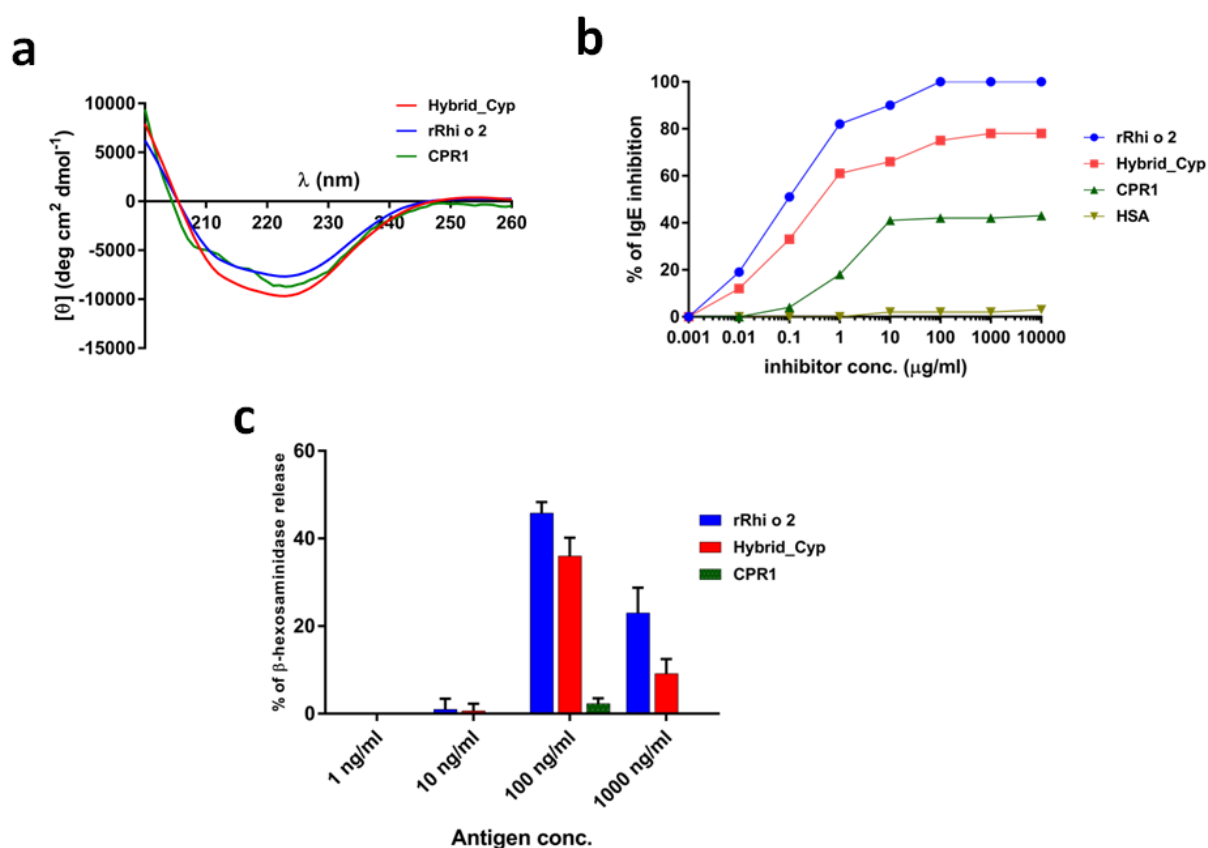


Fig 9: Increasing IgE-reactivity and allergenic activity of CPR1 by epitope grafting. (a) CD spectra of 5 μ M each of rRhi o 2, rCPR1, and rHybrid_Cyp at 25°C showing molar ellipticities (y axis) against wavelengths (nm in x axis). (b) ELISA inhibition showing IgE-binding capacity of CPR1 and Hybrid_Cyp. rRhi o 2 was incubated with a pool of 4 patients' sera inhibited with increasing concentrations of test antigens (x axis) and percentages of inhibition of IgE-binding (y axis) to immobilized rRhi o 2 was calculated. Sera mixed with rRhi o 2 and HSA for auto-inhibition and negative control respectively. (c) RBL-assay showing allergenic activity of Hybrid-Cyp *versus* CPR1 in which increasing concentrations of test antigens or rRhi o 2 as positive control (x axis) were used to stimulate RBL cell line passively sensitized with patients' IgE-antibodies. Bar graph represents means with SDs (error bars) for sera from six patients' sera

Molecular Characterization of a Fungal Cyclophilin Allergen Rhi o 2 and Elucidation of Antigenic Determinants Responsible for IgE-Cross reactivity

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Running title: *Characterization and epitope analysis of Rhi o 2 allergen*

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Keywords: Allergen, allergy, cyclophilin, fungus, immunoglobulin E (IgE), epitope mapping.

Contents: Supplementary Fig. S1 to S4 and Supplementary table S1

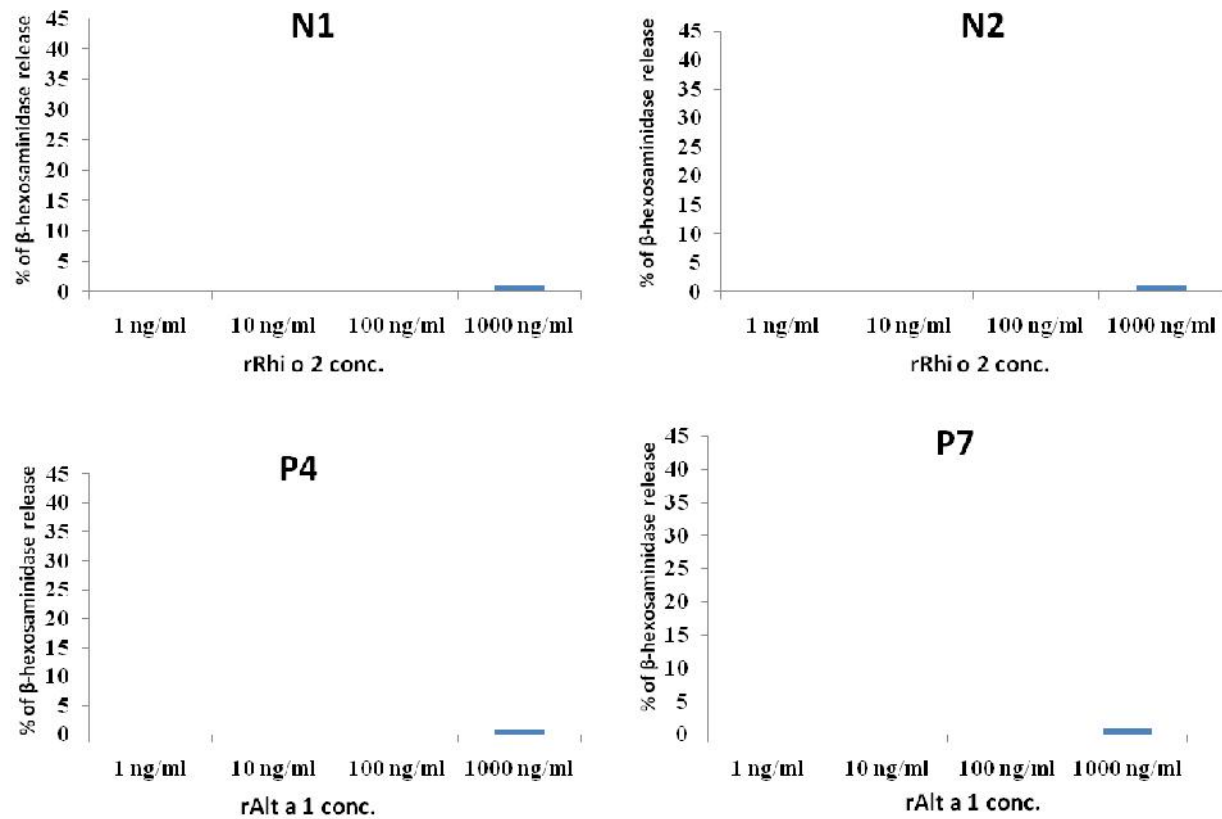


Fig S1: Control sets for the RBL assay. The RBL cells were either sensitized with non-atopic sera (N1 and N2) followed by challenge with rRhi o 2 or sensitized with IgE-antibodies of sera from 2 Rhi o 2-reactive patients (P4 and P7) followed by challenge with an unrelated fungal allergen rAlt a 1. In all the cases, the percentage of release (y-axis) was below 5%.

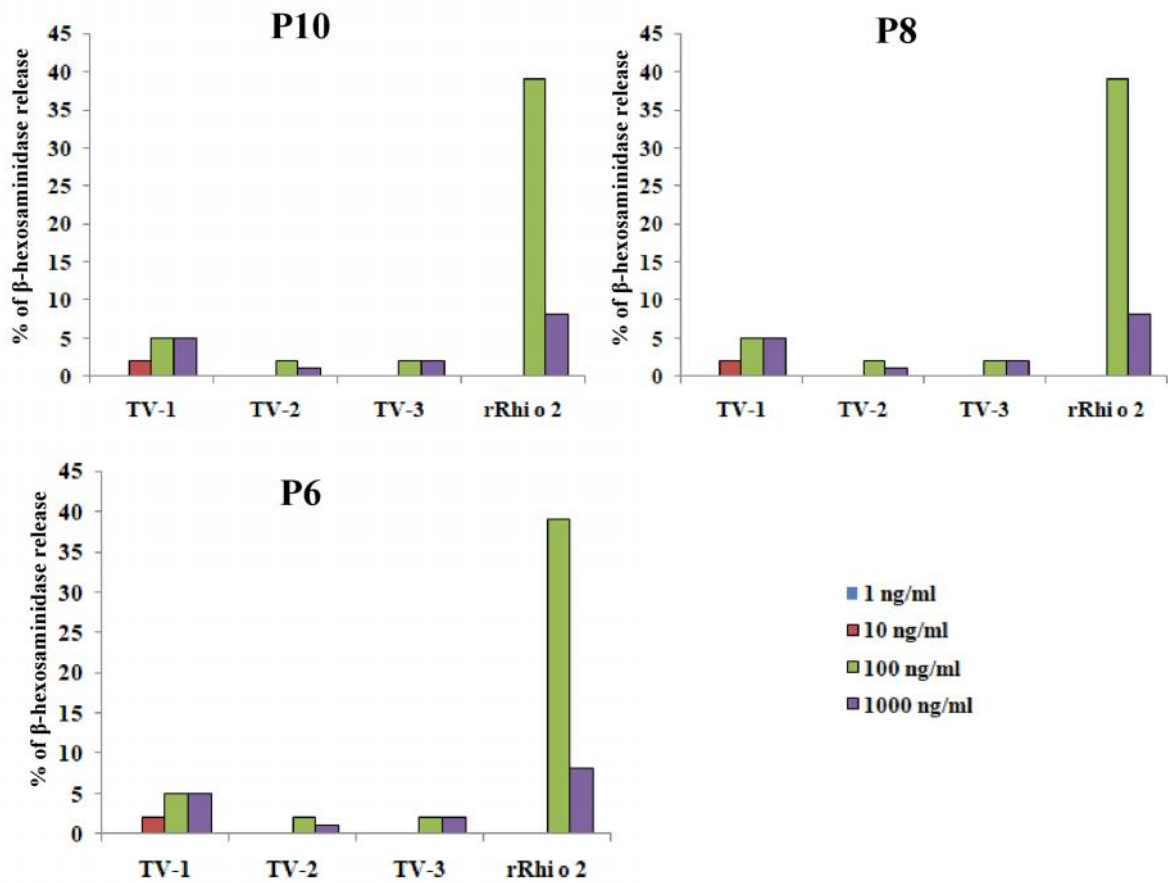


Fig S2: Comparison of allergenic activity of rRhi o 2-derived truncated versions: Increasing concentrations of either rRhi o 2 or each of its' IgE-reactive truncated versions (TV-1 to TV-3) (x axis) were used to stimulate the RBL cell line passively sensitized with IgE-antibody from 3 *R. oryzae* reactive sera to compare the β -hexosaminidase release (y axis) from stimulated RBL cells.

Rhi o 2	-----MSNPKVFFDVSA-----NSKPLGRIVMELRADVVPQTAENFRALCTGEKGF---	47
Asp f 11	FHQTPSAMSQV***VEYAPVGTAEKV*RIVFN*FDKDV*KTA***E**KRPA*E----	57
Bet v 7	-----MASNPKV***MEV-----GGQPV*RIVME*YADTT*RTAE***A**TGEK*N* RSG	51
Cat r 1	-----MPNPRV***MSV-----GGQPA*RIVME*FADTT*RTAE***A**TGEK*T* RSG	50
Sola l 5	-----MANPKV***LTI-----GGAPA*RVVME*FADTT*KTA***A**TGEK*V* KMG	50
Der f 29	-----MALPRV***IAA-----DNQPL*RIVIE*RSVV*KTA***A**TGEK*F*----	47
Asp f 27	-----MVVKT***ITI-----DGQPA*RITFK*FDEVV*KTVE***A**TGEK*F*----	46
Mala s 6	-----MSNV***ITK-----NGAPL*TIKEK*FDDVV*KTAA***A**TGEK*F*----	45
Rhi o 2	----YKGSFHRVPEF <u>ML</u> QGGDFTNHNGTGGKSIYG NKFRDENFTLKHTGPGDLSMANA	103
Asp f 11	----YREST***I**N**I*****RG*****R*****DK*A****SRK*DKK*IL*****	113
Bet v 7	KPLHY KKSS***V**G**C*****AG*****E*****AK*A****IKK*TGP*IL*****	111
Cat r 1	KPLHY KDSS***V**G**C*****AG*****E*****AK*A****IKK*TGP*IL*****	110
Sola l 5	KPLHY KGST***V**G**C*****AG*****E*****AK*N****VKK*TGP*IL*****	110
Der f 29	----FKSSS***I**N**I*****NH*****K*****NK*A****TLQ*TGP*IM*****	103
Asp f 27	----YKGSS***I**Q**L*****KG*****K*****DR*P****QLK*DKP*LL*****	102
Mala s 6	----YAGSH***V**D**L*****AG*****K*****AK*A****QLK*NKP*LL*****	101
Rhi o 2	GPNTNGSQFEITTIKCSWLDGKHVVFGRVTEG--MDVVQNIESLGSPN GTPR--AKIIID	159
Asp f 11	*PN*****T*AVTS**D*K*****E*ADEKSYSV**E**AL**SS*S ^V RSNTRPKIV	173
Bet v 7	*PG*****C*AKTE**D*K*****Q*VEG--LDI**A**KV**SS*RTS--KPVVVA	167
Cat r 1	*PN*****C*AKTE**D*K*****Q*VEG--MDV**A**KV**SS*RTA--KKVVVE	166
Sola l 5	*PG*****C*AKTE**N*K*****Q*VEG--MDV**K**AV**SS*RCS--KPVVIA	166
Der f 29	*PN*****T*VKTT**D*K*****S*VEG--MDI**K**SY**QS*KPS--KKVTIA	159
Asp f 27	*KN*****T*VVTs**D*A*****E*EDG--MDL**K**SY**AS*TPK--KKITIA	158
Mala s 6	*PN*****T*VVTs**D*K*****E*IDG--MNV**A**AE**GS*KPR--SRIEIA	157
Rhi o 2	NCGQL- 164	
Asp f 11	N**EL- 178	
Bet v 7	D**QLS 173	
Cat r 1	D**QLS 172	
Sola l 5	D**QL- 171	
Der f 29	N**QL- 164	
Asp f 27	D**QL- 163	
Mala s 6	K**VC- 162	

Fig S3: Multiple Sequence Alignment of Rhi o 2 with 7 other cyclophilins such as Asp f 11 and Asp f 27 from *Aspergillus fumigatus*, Bet v 7 from birch pollen, Cat r 1 from periwinkle pollen, Sola l 5 from tomato, Der f 29 from house dust mite, and Mala s 6 from skin colonizing yeast. All these cyclophilins are reported as allergens and enlisted in WHO/IUIS allergen database. Identical residues in the aligned sequences are shown as asterisks and gaps as dashes with respect to the reference sequence Rhio 2 in the top. N⁸¹-N¹⁴⁹ fragment of Rhi o 2 is shown in red. The 7 residue loops specific for plant cyclophilins are in green. The active site residues responsible for PPIase activity are underlined.

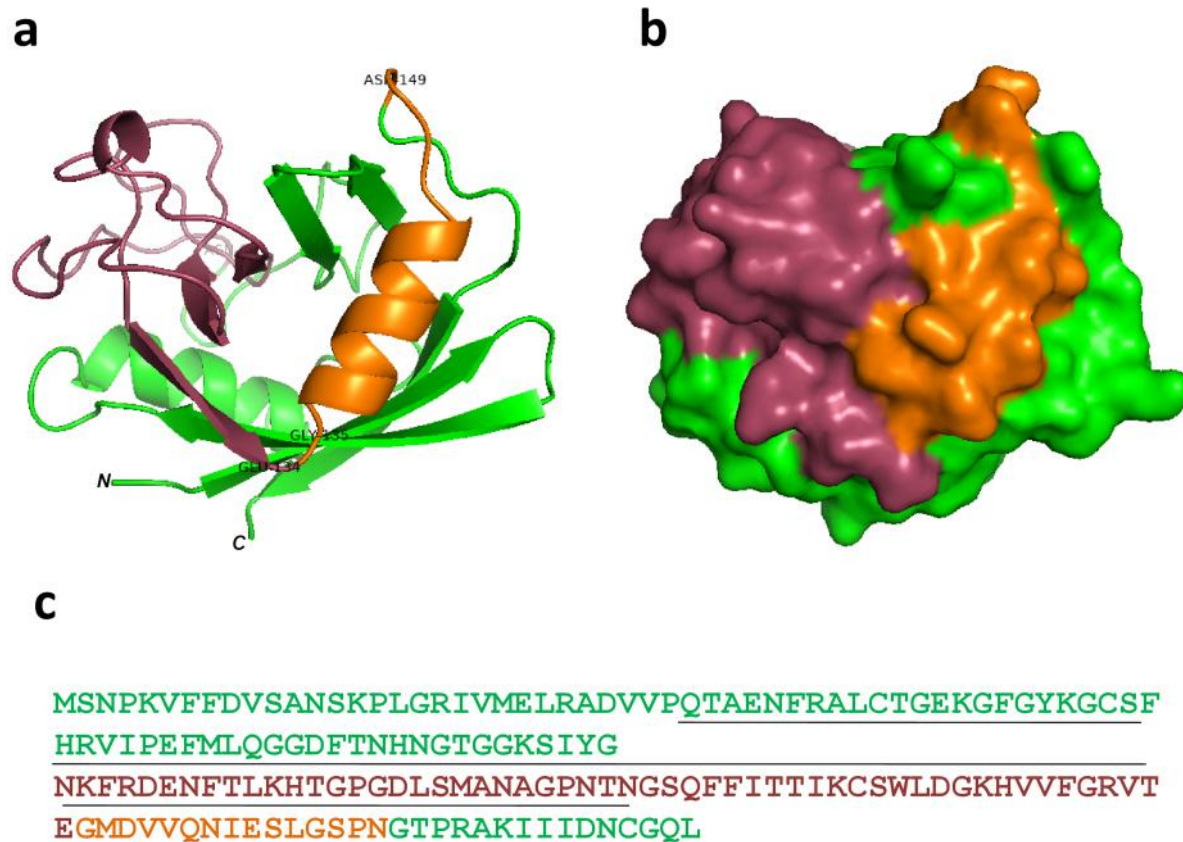


Fig S4: Rhi o 2 tertiary structural model and location of N⁸¹-N¹⁴⁹ fragment. (a) Ribbon diagram and (b) Surface representation of Rhi o 2 homology model shown in green. The C-terminal 15 solvent-accessible residues (G¹³⁵-N¹⁴⁹) of the N⁸¹-N¹⁴⁹ fragment are shown in orange while rest of the N⁸¹-N¹⁴⁹ fragment is in magenta. (c) For convenience of understanding, the amino acid sequence of Rhi o 2 and N⁸¹-N¹⁴⁹ fragment is shown using the same color code as used in the structural models. Sequence of TV-3 is underlined.

Table S1: Clinical and demographic details of human subjects participated in the study.

Subject ID	Age/Sex	tIgE (IU/ml)	sIgE for RCE (kU/ml)	sIgE for rRhi o 2 (A_{405})	Symptoms	Other major sensitizers in ImmunoCAP
P1	32/M	652.00	17.80	0.854	BA	HDM+AF+SF
P2	41/M	365.88	12.80	0.603	BA	HDM+AF+TM
P3	28/M	1874.00	60.20	1.241	BA+AR	HDM+AF
P4	32/F	581.06	24.90	0.678	BA	HDM+AF+SF
P5	52/F	453.88	17.35	0.570	BA	HDM+AS+MK
P6	62/M	1280.23	57.80	0.998	BA	HDM+LG+EG+CK
P7	38/M	453.69	36.00	0.791	BA	HDM+AF+CK
P8	36/F	872.00	47.05	1.109	BA+AR	HDM+SF+PO
P9	55/M	367.00	19.35	0.587	BA+AR	HDM+AF
P10	25/F	635.20	23.88	0.594	BA	HDM+AF
E1	26/F	792.30	25.41	0.608	BA	HDM+SF+AS+GP
E2	23/M	803.20	48.90	1.184	BA	HDM+CT+LG
N1	24/F	51.00	0.08	0.118	<i>ns</i>	No
N2	28/M	64.10	0.04	0.103	<i>ns</i>	No
ARS	<i>nk</i>	341.08	0.09	0.121	BA	Only <i>Alternaria</i> spp.

FOOTNOTE for table S1

tIgE was estimated by Human IgE sandwich ELISA kit (Abcam) and sIgE by direct ELISA as already described earlier (References 33 and 36 of the main manuscript).

Abbreviations used: tIgE, total IgE; sIgE, specific IgE; RCE, *Rhizopus* crude extract; A_{405} , absorbance of ELISA plate at 405 nm; *nk*, not know; *ns*, no symptoms; BA, bronchial asthma; AR, atopic rhinitis; HDM, house dust mite; AF, *A. fumigatus*; SF, sea food; TM, tomato; MK, milk; LG, legumes; CK, cockroach; PO, *Penicillium oxalicum*; AS, *Altstonia scholaris* pollen; GP, grass pollen; CT, cotton;