

## Original article

# Nuclear transport factor 2 represents a novel cross-reactive fungal allergen

**Background:** Ubiquitously occurring moulds are important allergenic sources known to elicit IgE-mediated allergic diseases and to share cross-reactive allergens. Limited information is available about the molecular structures involved in cross-reactivity. We aimed to clone and characterize cross-reactive mould allergens.

**Methods:** Phage surface-displayed *Alternaria alternata* and *Cladosporium herbarum* cDNA libraries were screened using sera from *Aspergillus fumigatus*-sensitized patients. Inserts encoding putative allergens were sequenced, and recombinant proteins used to demonstrate cross-reactivity by inhibition experiments and skin test. Three-dimensional homology models of cloned putative nuclear transport factor 2 (NTF2) were constructed based on known NTF2 structure to corroborate the functional and structural properties of the novel allergens.

**Results:** After six rounds of affinity selection, the libraries were enriched for clones displaying allergens. Sequencing of inserts showed that some clones derived from *Alternaria alternata* and *Cladosporium herbarum* contain open reading frames predicting proteins of 124 and 125 amino acids corresponding to NTF2. The recombinant proteins were able to bind and cross-inhibit IgE binding and to elicit type I skin reactions in mould-sensitized individuals, demonstrating the allergenicity of the proteins.

**Conclusions:** NTF2 represents a novel cross-reactive fungal allergen as demonstrated by sequence homology, three-dimensional modelling, inhibition experiments and skin test reactivity.

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*Alternaria alternata* (*A. alternata*) and *Cladosporium herbarum* (*C. herbarum*) belong to the most important allergenic moulds ubiquitously occurring in all continents. Whereas *A. alternata* grows preferentially in warm and humid environments, *C. herbarum* dominates in cooler climates (1, 2). Both moulds are known to elicit antibody-dependent allergic diseases such as IgE-mediated rhinitis, conjunctivitis and asthma, according to the new mechanism-based classification of allergic reactions (3). Several fungal IgE-binding molecules, including allergens of *A. alternata* and *C. herbarum* have been cloned and characterized to date (4–7). However, cross-reactivity between *A. alternata* and *C. herbarum* has been demonstrated by RAST and immunoblot inhibition experiments (8) but not between these two moulds and *Aspergillus fumigatus* (*A. fumigatus*) (8), the most important allergenic mould for humans. *Aspergillus fumigatus* is the aetiological agent responsible for many respiratory disorders ranging from saprophytic colonization of the lungs, aspergilloma, IgE-mediated allergic rhinitis and sinusitis, hypersensitivity pneumonitis, IgE-mediated allergic

asthma and allergic bronchopulmonary aspergillosis (ABPA) to invasive aspergillosis (for review see (9)). ABPA is a severe pulmonary complication resulting from hypersensitivity to *A. fumigatus* in patients suffering from allergic asthma (10–12) or cystic fibrosis (CF) (13). However, sera from our ABPA patients with high specific IgE levels to *A. fumigatus* often show specific IgE raised against other moulds, including *A. alternata* and *C. herbarum*. To provide a rational approach for the direct selection of cross-reactive structures we examined cross-screening of phage surface displayed libraries from *A. alternata* and *C. herbarum* with serum IgE of patients suffering from ABPA. Phage surface display of cDNA libraries has been successfully applied to identify allergen repertoires from *A. fumigatus* (7), *Malassezia furfur* (14), *Coprinus comatus* (15), peanuts (16, 17) and mites (18), and allows fast cloning of cDNAs encoding IgE-binding proteins (19, 20). Here we describe the selection and characterization of two highly similar *A. alternata* and *C. herbarum* proteins from phage surface display libraries enriched with sera of patients suffering from ABPA.

Data base searches and homology modelling showed that both fungal sequences represent putative nuclear transport factor 2 (NTF2), a novel cross-reactive IgE-binding structure able to elicit positive skin prick test *in vivo*. Three-dimensional homology models of the fungal proteins based on the solved crystal structure of rat NTF2 (21), which is identical to human NTF2 (22, 23), were generated and revealed similar folds with a high conservation of the amino acids involved in the formation of the hydrophobic cavity of NTF2. *Alternaria alternata* and *C. herbarum* NTF2 show a sequence identity of 43% and 39%, respectively, with the human NTF2 (22, 23), yet without evidence for *in vitro* or *in vivo* IgE reactivity of the human protein.

## Material and methods

### Strains, plasmids and reagents

The *E. coli* strains XL1-Blue (24) and M15 (25) were used to generate cDNA libraries displayed on the surface of the filamentous phage M13 and for the production of recombinant proteins, respectively. The pJuFoII phagemid is a derivative of pJuFo (26) where the *Xba*I and *Kpn*I restriction sites were substituted by *Eco*RI and *Xho*I restriction sites to allow direct subcloning of inserts from pre-made  $\lambda$ -Zap libraries (20). Plasmid pQE30 (27) was modified to eliminate the His<sub>6</sub>-tag as described below. Helper phage VCSM13 was from Stratagene (Amsterdam, the Netherlands). DNA-modifying enzymes were from Roche Diagnostics (Rotkreuz, Switzerland) and were used as recommended by the manufacturer. Chemicals were of the highest available grade and were obtained from Fluka (Buchs, Switzerland) or Sigma (St. Louis, MO) unless otherwise specified.

### Construction of cDNA libraries displayed on phage surface and biopanning

For the construction of phage surface displayed cDNA libraries from *A. alternata* and *C. herbarum*, 4  $\mu$ g pJuFoII (20) were restricted with *Eco*RI and *Xho*I and dephosphorylated. Two  $\mu$ g cDNA inserts, prepared from previously constructed  $\lambda$ -Zap expression libraries (6) by digestion with *Eco*RI and *Xho*I, were ligated to the restricted vector. Electrocompetent *E. coli* XL1-Blue cells were transformed with the ligation mixtures to generate primary libraries. Phage surface display libraries were generated by superinfection with VCSM13 helper phage (Stratagene) following published protocols (19, 20, 28). The libraries were selectively enriched for phage displaying IgE-binding proteins by consecutive rounds of biopanning in microtitre plates coated with serum IgE from individuals with IgE-mediated allergy to *A. fumigatus* (19). Sera from patients suffering from ABPA were selected according to case history, specific IgE to *A. fumigatus*, *A. alternata* and *C. herbarum* determined by radioallergosorbent test (RAST), and skin test reactivity to commercial mould extracts (Table 1). After six rounds of affinity enrichment, a wide variety of phage binding to human serum IgE was isolated. Enriched cDNAs differing in length as shown by restriction analysis were sequenced using the ABI PRISM dRhodamine terminator cycle sequencing kit on an automated sequencer (ABI 377, Perkin-Elmer, Foster City, CA). Homology searches were performed with BLAST and the Genetics Computer Group program FASTA (29). Several clones derived from

Table 1. Serological characteristics of the patients' sera selected for affinity enrichment of cDNA libraries displayed on phage surface

| Patient no. | Age [years] | Sex | <i>A. fumigatus</i> RAST [kU/L] (RAST class) | <i>A. alternata</i> RAST [kU/L] (RAST class) | <i>C. herbarum</i> RAST [kU/L] (RAST class) |
|-------------|-------------|-----|----------------------------------------------|----------------------------------------------|---------------------------------------------|
| 1           | 28          | F   | 11.3 (2)                                     | 42.8 (3)                                     | 25.2 (3)                                    |
| 2           | 58          | F   | 1394.0 (5)                                   | 20.4 (3)                                     | 14.5 (2)                                    |
| 3           | 64          | M   | 137.0 (5)                                    | 47.8 (3)                                     | 26.8 (3)                                    |
| 4           | 41          | F   | 50.2 (4)                                     | 12.9 (2)                                     | 11.7 (2)                                    |
| 5           | 45          | F   | 99.3 (4)                                     | 5.89 (2)                                     | 6.61 (2)                                    |
| 6           | 25          | M   | 17.8 (3)                                     | 7.32 (2)                                     | 25.7 (3)                                    |
| 7           | 31          | M   | 63.2 (4)                                     | 5.69 (2)                                     | 9.51 (2)                                    |
| 8           | 44          | F   | 61.7 (4)                                     | 16.5 (2)                                     | 5.35 (2)                                    |

both libraries revealed significant homology with nucleotide sequences encoding nuclear transport factor 2 (NTF2, Fig. 2).

### Cloning, production and purification NTF2

The complete cDNAs encoding the putative *A. alternata* (aaNTF2) and *C. herbarum* (chNTF2) NTF2 proteins were PCR-amplified from the original phagemid clones using the following primers: aaNTF2: 5'-primer 5'-C GGA ATT CAT TAA AGA GGA GAA GGA TCC ATG TCG GAC TTC AAC GCA ATC G-3'; 3'-primer 5'-CC AAGCTT CTA CGC GGC GGG GTA TAC GAG ACG G-3'. chNTF2: 5'-primer: 5'-C GGA ATT CAT TAA AGA GGA GAA GGA TCC ATG TCG GAC TTC AAC GCA ATC G-3'; 3'-primer: 5'-CC AAG TCC TTA CAC TTC ACT ATC AGC CTT CCC-3'. PCR cycling conditions were 94°C for 60 s, 57°C for 60 s, and 72°C for 60 s for 30 cycles, followed by a terminal extension cycle at 72°C for 10 min. The amplification products were purified over QIAquick spin columns (Qiagen, Chatsworth, CA), digested with *Eco*RI and *Hind*III, and ligated into appropriately restricted vector pQE30, thereby eliminating the vector's His<sub>6</sub>-tag and reconstituting the ribosomal binding site with primer-derived oligonucleotides. The ligation products were transformed into *E. coli* strain M15/pREP4 electrocompetent cells (27). Transformants containing aaNTF2 and chNTF2 were grown in liquid culture to verify the nucleotide sequences and used to produce mature recombinant proteins as described (27). The sequence encoding human NTF2 (23) was amplified from a commercial lymphoma U937 human lung cDNA library (Stratagene) using the following primers: 5'-GA AGA TCT ATG GGA GAC AAG CCA ATT TGG-3' and 5'-CC AAG CTT TCA GCC AAA GTT GTG CAG GG-3'. Purified PCR products were restricted with *Bgl*II and *Hind*III and cloned into *Bam*HI/*Hind*III-restricted chNTF2 vector to obtain hsNTF2. Batch cultures of 1.2 l were grown as described (27), harvested by centrifugation at 4500 g for 15 min at 4°C and frozen at -20°C. Recombinant fungal proteins were solubilized by thawing and resuspension of cell pellets in 20 ml 8 M Urea, 10 mM Tris, 1.0 mM EDTA, pH 8.0 followed by centrifugation (17 500 g, 15 min, 4°C). After sterile filtration the cleared lysates were diluted in 500 ml TE (pH 8.0) and ammonium sulphate was added to 35% saturation. Precipitated contaminants were removed by centrifugation at 50 000 g for 30 min at 4°C. In a second step the precipitate obtained from a 35–70% ammonium sulphate fraction was resuspended in 5 ml of TE (pH 8.0) saturated to 25% with ammonium sulphate. The suspension was clarified by centrifugation (50 000 g, 15 min, 4°C), dialyzed against TE (pH 8.0) and applied to a 150  $\times$  10 mm DEAE Sepharose Fast Flow column (Amersham Pharmacia Biotech, Uppsala, Sweden). After extensive washing

with TE (pH 8.0), a linear gradient of 0–250 mM NaCl in TE (pH 8.0) was used to elute the fungal NTF2 proteins. Homogeneous aaNTF2 eluted at 80–90 mM NaCl, and chNTF2 at 150–155 mM NaCl. These fractions were dialyzed against PBS containing 0.5 mM EDTA. Human NTF2 was purified as described (21, 30). The purified proteins were biotinylated by incubation with a two-fold molar excess of sulphosuccinimidyl-6-(biotinamido)-hexanoate (Pierce, Rockford, IL) for 4 h at room temperature. Hydrolyzed reactant was removed by extensive dialysis against TBS, containing 0.5 mM EDTA. Protein concentrations were determined on the basis of the calculated  $\epsilon_{280}$  coefficients (31). SDS-PAGE analysis of the proteins was performed on 4–20% Tris–Glycine polyacrylamide gels (Invitrogen, Amsterdam, the Netherlands).

Homology modelling and structural alignment

Models and structural alignments of the two fungal NTF2 were generated using the SWISS-MODEL automated comparative protein modelling server (<http://www.expasy.ch/swissmod/SWISS-MODEL.html>) (32). The models are based on the structure of NTF2 from rat (PDB accession number 1OUN) (21), which shares 100% sequence identity with the human NTF2 (SWISS-PROT accession number P13662) (22, 23).

Titration of allergen-specific serum IgE and competition ELISA

For allergen-specific IgE-titrations Nunc-Immuno Plates (Maxisorp Surface) were coated overnight at 4°C with 5 µg/ml avidin in PBS, washed and blocked with 2% (w/v) skimmed milk powder in PBS containing 0.1% Tween-20 (buffer A). After washing, 5 µg/ml of the respective biotinylated NTF2 in buffer A was added to the wells followed by incubation for 1 h at room temperature. After washing with PBS containing 0.1% Tween-20 (buffer B), sera were diluted 1 : 8 in buffer A, serially diluted two-fold throughout the plate and incubated for 2 h at 37°C. IgE binding was visualized by incubation with mAb TN-142 mouse anti-human IgE mAb (1 mg/ml) diluted 1 : 6500 in buffer A and detected with alkaline-phosphatase-conjugated goat anti-mouse IgG H + L chain (Pierce) in buffer A as described (33). Competition ELISAs between the different NTF2 proteins were performed on ELISA plates coated with avidin as described, using non-biotinylated NTF2 proteins and recombinant rAsp f 1 protein as negative control (34). Percentage of competition was calculated from the absorbency of the serial dilutions containing rAsp f 1 in fluid phase set as 0% competition. The same procedure was used to determine the inhibition between *A. fumigatus* and *C. herbarum* extracts using ELISA plates coated with *A. fumigatus* extract (10 µg/ml) and BSA as a negative control.

For graphic display, the measured absorbencies were plotted against the antigen concentration in fluid phase.

Western blotting

Recombinant proteins were separated on 4–20% Tris–Glycine polyacrylamide gels (Invitrogen) under reducing conditions, transferred to Immobilon-P<sup>SO</sup> polyvinylidene-fluoride membranes (Millipore, Bedford, MA) at 10 mA/cm<sup>2</sup> and processed as described (34). After incubation with patients' sera diluted 1 : 10 in buffer A, binding of specific IgE was detected by incubation with TN-142 mouse anti-human IgE mAb. Western blots were developed with the ECL Plus system (Amersham Pharmacia Biotech, Rockford, IL) using horseradish peroxidase-conjugated goat anti-mouse IgG as a detection system (34).

Skin tests with recombinant *A. alternata* and *C. herbarum* allergens

Skin prick tests (SPT) were performed as described elsewhere (35). Commercially available allergen extracts from *A. alternata* and *C. herbarum* (ALK, Copenhagen, Denmark) were used in the provided dilution (100 000 SQE/ml). Recombinant allergen solutions for skin testing were prepared as described (33). All test solutions were applied in duplicate in inverse order on the volar forearm of the patients. Skin test reactions were recorded after 15 min and considered positive with a wheal > 3 mm in the absence of reaction to the saline control (35). The study design was approved by the ethical committee of the University of Zurich. A full oral and written explanation of the procedure was given to the participants, and their written consent was obtained before testing.

Results

Competition ELISA with fungal extracts

Although RAST inhibition experiments between *A. fumigatus* and *C. herbarum* extracts failed to show relevant cross-reactivity between the moulds in an earlier study (8), a RAST survey of sera from patients suffering from ABPA revealed the presence of specific IgE raised against both moulds (Table 1). Inhibition experiments between *A. fumigatus* and *C. herbarum* extracts coated to solid phase using sera from two of these patients clearly

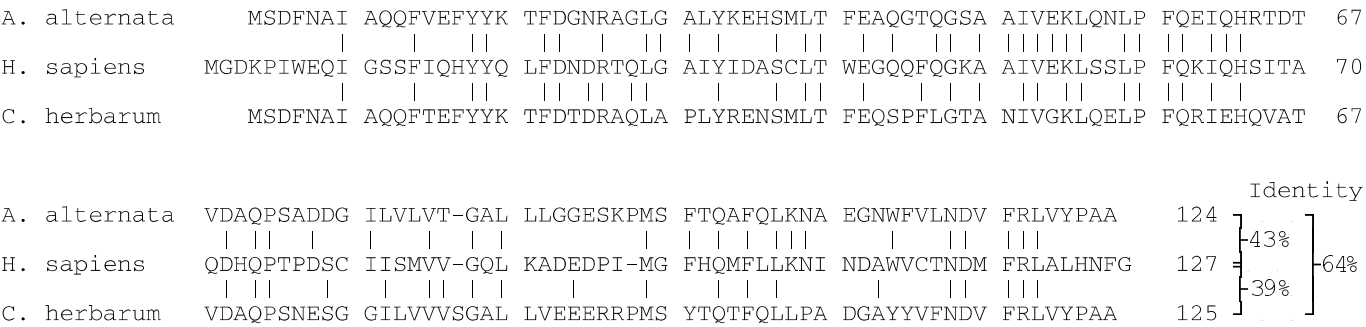


Figure 1. Alignment of the deduced amino acid sequences of *A. alternata*, human and *C. herbarum* NTF2. Identical amino acids are marked by vertical bars, gaps by dots. Numbers on the right side indicate the residue numbers, without gaps, starting at the NH<sub>2</sub>-terminal methionine residues.

showed cross-reactivity between the extracts (Fig. 1). Therefore, we decided to cross-screen phage surface displayed cDNA libraries derived from *C. herbarum* and *A. alternata* with a serum pool of ABPA patients with the aim to isolate cross-reactive allergens.

#### Library construction and selection of putative fungal NTF2 by phage surface display

We have constructed cDNA libraries from *A. alternata* and *C. herbarum* in the pJuFoII phagemid (20) containing  $1.0 \times 10^6$  and  $8.9 \times 10^8$  independent transformants, respectively, which were used to generate libraries displayed on the surface of filamentous phage M13 as described (19, 36). The libraries were selectively enriched for phage displaying IgE-binding proteins by biopanning in microtitre plates coated with serum IgE from individuals suffering from ABPA. After six cycles of affinity selection and amplification, the phage population obtained was enriched for phage carrying inserts of different length potentially encoding IgE binding proteins. A sample of 14 single clones was subjected to restriction analysis and the nucleotide sequences of inserts differing in length were determined using vector-derived primers. The deduced amino acid sequences showed that each clone derived from the *A. alternata* and *C. herbarum* libraries contained inserts revealing strong homology with nucleotide and amino acid sequences of NTF2 (Fig. 2). The clone encoding chNTF2 (EMBL accession number AJ 493 279) contains an insert of 624 bp consisting of a coding region of 375 bp (bases 7–381) predicting a protein of 125 aa. The 3′-noncoding region has a polyadenylated tail of 23 bp. The clone encoding aaNTF2 (EMBL accession number AJ 493 280) contains an insert of 710 bp consisting of a coding region of 372 bp (bases 52–423) predicting a protein of 124 aa. The 3′-noncoding region has a polyadenylated tail of 19 bp. The correct open reading frames were identified by start and stop codons in the nearly correct consensus context (37) and by multiple sequence comparisons (Fig. 2). The amino acid sequences deduced from the cDNAs showed the highest sequence identity of 49–52% to yeast NTF2 (38), 39%–43% to human NTF2 (22, 23) and shared 64% identity with each other. Apparently, the fungal NTF2 display a similar degree of sequence identity to NTF2 from a wide variety of phylogenetically distant organisms.

#### Homology modelling

Homology modelling of the three-dimensional structures of aaNTF2 and chNTF2 was based on the solved three-dimensional structure of rat NTF2 (SWISS-PROT accession number P13662; PDB accession number 1OUN; Fig. 3A) (21) and yielded conformations that were similar to the template structure. Conformational differences of the protein backbones were found in the loop between  $\beta$ -strand 4 and  $\beta$ -strand 5 in both proteins

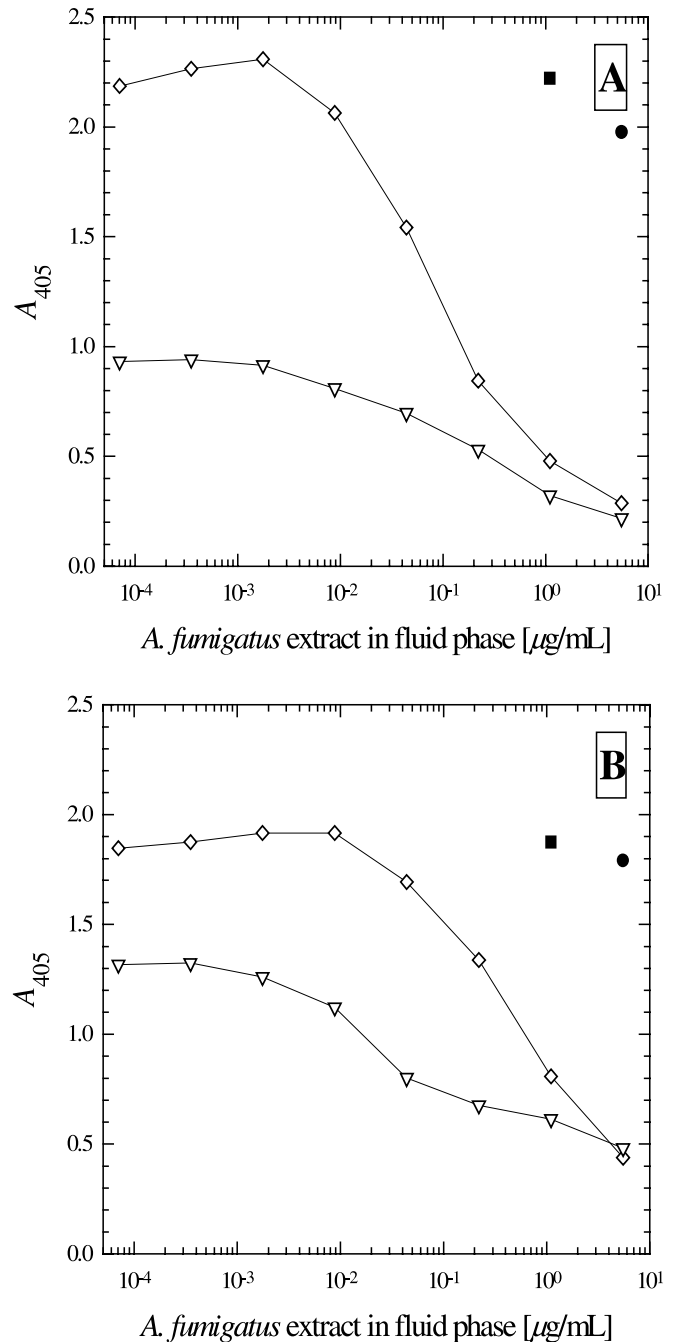


Figure 2. Cross-inhibition of IgE binding between *A. fumigatus* and *C. herbarum* extracts. Sera from two patients (◇, ▽) were pre-incubated with increasing concentrations of *A. fumigatus* extract and subsequently applied to ELISA plates coated with *A. fumigatus* (A) or *C. herbarum* (B) extract. *E. coli* extract (■) and extraction buffer (●) were used as a negative control.

and in the middle part of  $\beta$ -strand 4 of chNTF2 (Fig. 3B,C). The amino acids which form the base of the hydrophobic cavity were found to be highly conserved in all proteins (Fig. 3D–F). The amino acids which form the entrance and the upper part of the cavity which show few

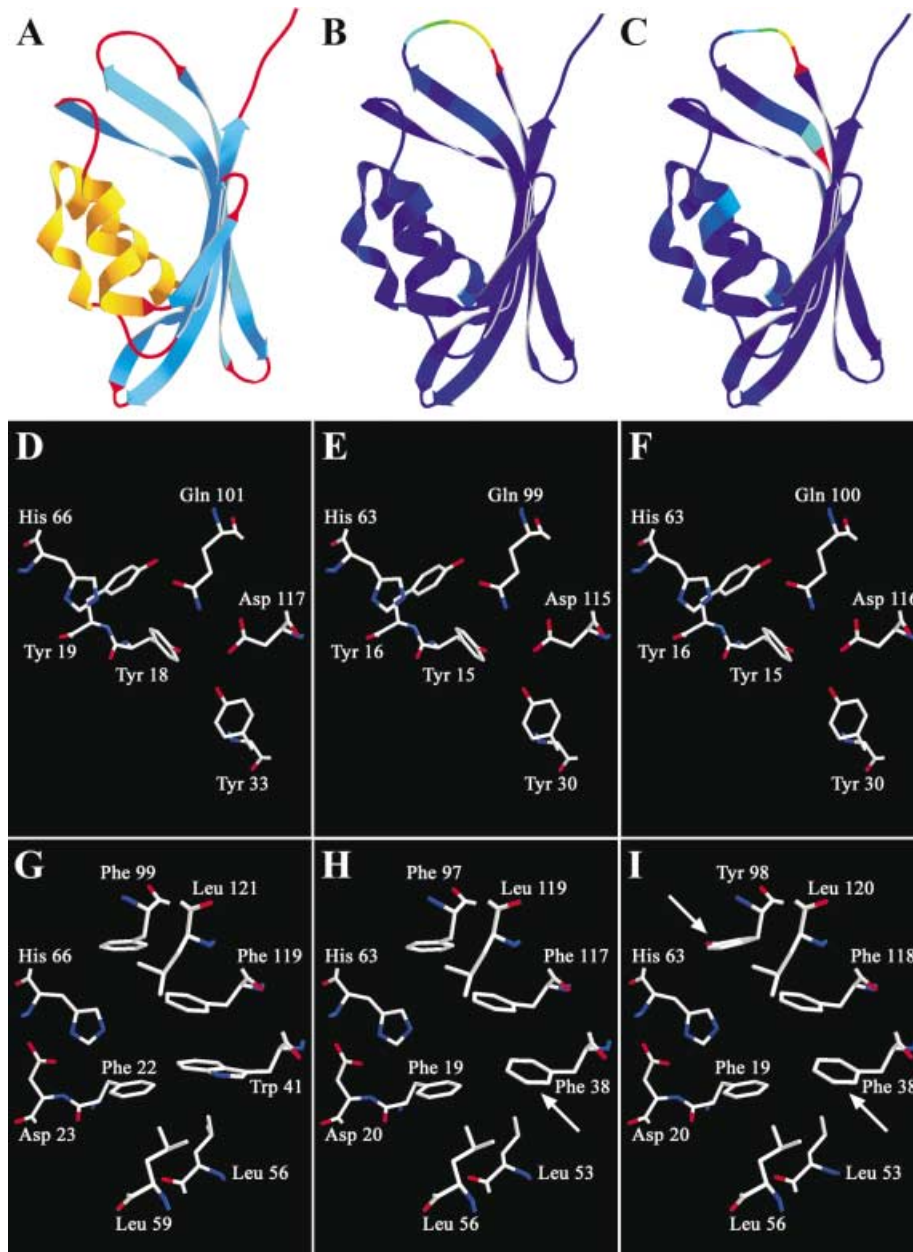


**Figure 3.** Three-dimensional homology models of putative *A. alternata* (B, E, H) and *C. herbarum* (C, F, I) NTF2 compared to the rat NTF2 monomer (A, D, G). (A) NTF2 from rat depicted according to (21). Helices are coloured yellow, sheets blue and other secondary structure elements red. (B, C) Deviations of the fungal protein backbones are coloured according to the root mean square distances from dark blue (lowest) to red (highest). (D–F) The polar groups of the hydrophobic cavity are highly conserved in all NTF2. (G–I) Amino acid residues forming the entrance and upper part of the cavity. Conservative exchanges found in the fungal NTF2 are indicated with white arrows.

conservative exchanges, i.e., Trp 41 → Phe 38 found in both fungal proteins and Phe 99 → Tyr 98 found only in chNTF2 (Fig. 3,A–C). Therefore we conclude that the fungal sequences, structurally as well as functionally, represent NTF2 as shown in the models in Fig. 3 depicted according to Bullock et al. (21). The sequence identity between *A. alternata* and *C. herbarum* NTF2 to human NTF2 was 43% and 39%, respectively (Fig. 2).

#### Expression and purification of putative fungal NTF2

The complete cDNA sequences encoding the putative *A. alternata*, *C. herbarum* and the human NTF2 proteins were expressed in *E. coli* as mature proteins without fusion tags and purified as described in Material and methods, resulting in yields of 94, 88 and 50 mg of pure protein per litre culture. For binding assays, the proteins

were biotinylated with a two-fold molecular excess of EZ-Link™ Sulfo-NHS-LC-Biotin. The recombinant proteins as well as the biotinylated forms thereof were virtually pure when analysed by SDS-PAGE and Coomassie blue staining (Fig. 4). The estimated molecular masses determined by regression analysis using the marker proteins as standard were in good agreement with the calculated values of 13.7 kDa, 14.2 kDa and 14.5 kDa for the *A. alternata*, *C. herbarum* and human NTF2, respectively.

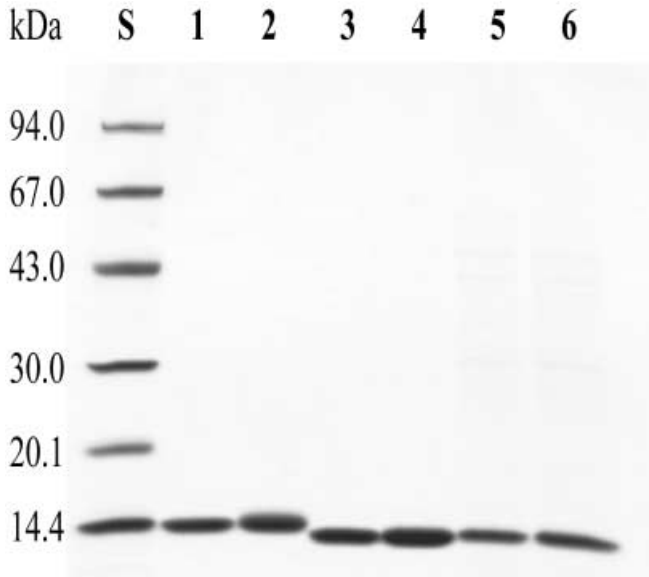


Figure 4. SDS-PAGE analysis of the purified recombinant proteins. Lane S, molecular mass standard (kDa). Lanes 1, 3 and 5 *C. herbarum*, *A. alternata* and *H. sapiens* NTF2. Lanes 2, 4 and 6 show the purity of the biotinylated forms of the proteins shown in lanes 1, 3 and 5.

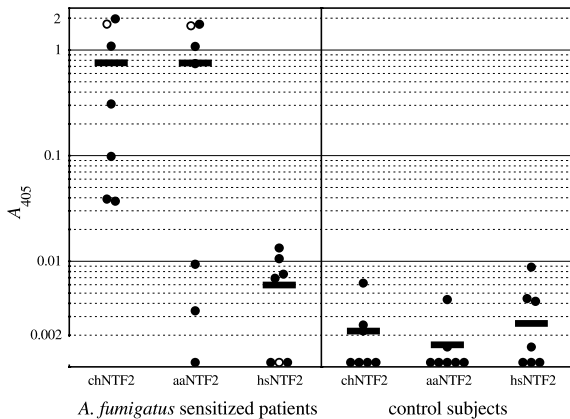


Figure 5. Specific IgE against *C. herbarum* (ch), *A. alternata* (aa) and human (hs) NTF2 in sera from individuals sensitized to *A. fumigatus* (solid circles, left), *C. herbarum* (open circles) and control individuals. Mean values are indicated by bars.

Specific IgE binding, competition ELISA, and Immunoblots

The IgE-binding capacity of the purified recombinant proteins was determined by ELISA. Specific IgE against chNTF2 and aaNTF2 ( $A_{405} \geq 0.1$ ) could be detected in at least four out of 480 patients sensitized to *A. fumigatus*, whereas IgE binding to hsNTF2 was negligible (Fig. 5). Furthermore we could identify serum from 1 out of 12 *C. herbarum* sensitized patients, which showed strong IgE binding to the fungal NTF2. As control, sera of healthy individuals were used, and showed IgE binding less than three times the background value. IgE binding to the fungal allergens could further be confirmed by Western analysis (Fig. 6).

In a further step we investigated whether the fungal allergens shared common IgE epitopes. Biotinylated aaNTF2 was immobilized on a solid phase via avidin and the binding of serum IgE from *A. fumigatus* sensitized patients was efficiently outcompeted by increasing amounts of non-biotinylated aaNTF2 and chNTF2 in the fluid phase, whereas hsNTF2 did not show any competitive effect (Fig. 7). A structurally unrelated allergen,

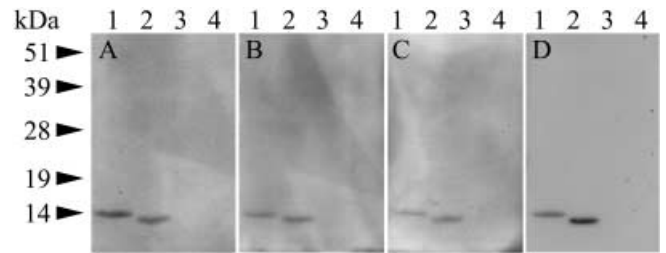


Figure 6. IgE binding of chNTF2 (1), aaNTF2 (2) and hsNTF2 (3) in Western blotting with sera from four NTF2-sensitized individuals (A–D). In lane 4 10 µl *E. coli* lysate from an uninduced cell culture was applied as negative control, showing that the serum is free from IgE directed against *E. coli* proteins.

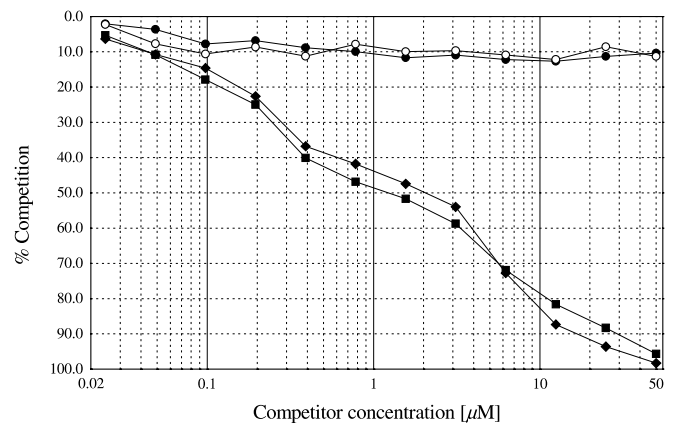


Figure 7. Inhibition of IgE binding to aaNTF2 coated on solid phase by aaNTF2 (◆), chNTF2 (■), hsNTF2 (●) and rAsp f 1 (○) as negative control in the fluid phase.

rAsp f 1 from *A. fumigatus*, was used as a negative control. These results show that both fungal NTF2s share common IgE-binding epitopes, whereas the structurally homologous human NTF2 lacks capability to bind IgE from the sera tested.

#### Skin prick test

The final demonstration of the allergenicity of a protein is its ability to elicit a type I skin reaction in sensitized individuals. We therefore performed a skin test in an individual sensitized to *C. herbarum* suffering from intermittent allergic rhinitis and asthma and showing specific serum IgE to the newly identified allergenic structure. Both chNTF2 and aaNTF2 sharing 64% sequence

identity showed strong skin reactivity (Fig. 8) in perfect accordance with the presence of allergen-specific IgE in the serum. No skin reactivity was observed with human NTF2, which was negative also in ELISA and Western blot, supporting earlier reports showing that proteins with a sequence identity of less than 50% generally do not cross-react (39).

#### Discussion

It is known that moulds show cross-reactivity in serological IgE determinations using extracts (8, 40). However, cross-reactivity between *A. fumigatus* and *C. herbarum* or *A. alternata* has never been demonstrated in the past (8). A RAST survey of sera from patients suffering from ABPA (Table 1) showed that these patients are sensitized to all three moulds, but this could be the result of different sensitizations against the single species. To test whether the extracts share IgE-binding structures, we performed competition experiments on plates coated with *A. fumigatus* or *C. herbarum* extract using extract from *A. fumigatus* as competitor in fluid phase. As shown in Fig. 1, a relevant reduction in the IgE-binding capacity of the sera could be detected, demonstrating that the extracts contain common IgE-binding structures. Therefore, we screened phage surface displayed cDNA libraries of *A. alternata* and *C. herbarum* with serum IgE from ABPA patients to isolate cross-reactive allergens. Among the phage population enriched for clones displaying IgE-binding molecules, two fungal sequences sharing 64% identity at amino acid level were detected. The *A. alternata* and *C. herbarum* inserts predict proteins of 124 and 125 amino acids, respectively, showing a high degree of sequence homology with proteins of the nuclear transport factor 2 family (Fig. 2). NTF2, a homodimeric protein with a molecular mass of 14.478 kDa per subunit (21) was originally identified as a cytosolic factor that stimulates the transport of nuclear localization sequence (NLS)-containing proteins into the nucleus of permeabilized mammalian cells (23, 41). NTF2 was shown to specifically bind RanGDP (38, 42) and to mediate its transport to the nuclear side of the nuclear pore complexes (43). In this study, putative fungal NTF2 of *A. alternata* and *C. herbarum* were cloned, sequenced and characterized. Homology modelling of the fungal proteins based on the solved three-dimensional structure of rat NTF2 yielded conformations similar to the template structure (Fig. 3). In particular, the amino acids which form the base of the hydrophobic cavity were found to be highly conserved as well as the amino acids which form the entrance and the upper part of the cavity. Based on sequence homology and modelling results, we conclude that the fungal sequences represent NTF2.

Fungal NTF2 was shown to correspond to a cross-reactive IgE-binding molecule able to bind IgE from sensitized patients in ELISA (Fig. 5) and Western blot

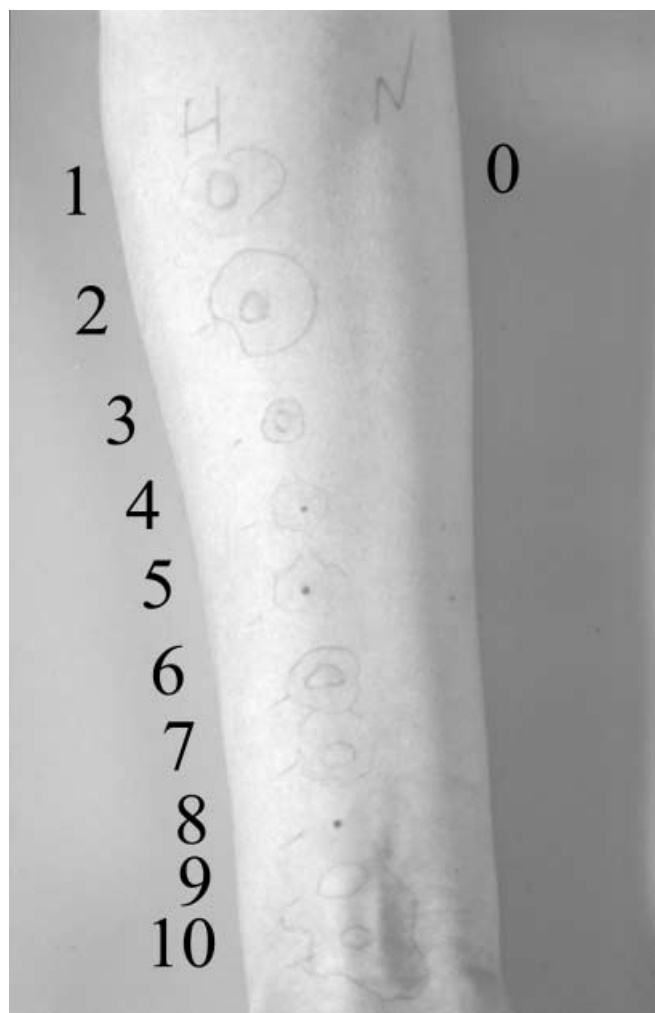


**Figure 8.** Skin prick tests with recombinant fungal allergens and fungal extracts in a patient sensitized to *C. herbarum*. 0.9% saline (0), 0.01% histamine dihydrochloride (1), *A. alternata* extract (2), Alt a 1 (3), Alt a 11 (4), Cla h 6 (5), aaNTF2 (6), chNTF2 (7), hsNTF2 (8), HCh-1 (9) and *C. herbarum* extract (10).

analysis (Fig. 6). Both molecules are able to compete for IgE binding in inhibition ELISA demonstrating the presence of shared B cell epitopes (Fig. 7). Interestingly, the new allergenic structure belongs to the class of intracellular, non-secreted proteins as reported for other allergens of these moulds, including ALDH, enolase and acidic ribosomal P<sub>2</sub> protein (6). Ribosomal P<sub>2</sub> protein of *A. fumigatus* (44) belongs to the class of phylogenetically highly conserved proteins and shares significant sequence homology to the corresponding human protein. Both the human and fungal protein are able to bind IgE from *A. fumigatus*-sensitized patients and to elicit strong type I skin reactions in the same individuals (44). As *A. alternata* and *C. herbarum* NTF2 share sequence identity of 43% and 39% with the human protein, we cloned, produced and tested human NTF2 for the ability to act as a cross-reactive autoallergen. In spite of the extended sequence homology, the human protein did not bind serum IgE from sera of individuals sensitized to fungal NTF2 and lacks the capability to elicit skin test reactions. We conclude that human NTF2, in contrast to

other phylogenetically conserved proteins (45), is not recognized by serum IgE from mould-sensitized individuals. Although the experimental results presented can not clearly distinguish between polysensitization to, and cross-reactivity between *A. alternata*, *C. herbarum* and *A. fumigatus*, NTF2 clearly represents a cross-reactive allergen produced by *A. alternata* and *C. herbarum*, recognized by sera from patients sensitized to *A. fumigatus*. Together with other cross-reactive allergens produced by moulds, NTF2 may contribute to reduce the number of structures required for a reliable diagnosis of fungal allergy which, at the present time, is based on fungal extracts. It is well recognized that the diagnosis of fungal allergy with mould extracts is far from reliable (46).

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