



Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



Short communication

Alternaria alternata TCTP, a novel cross-reactive ascomycete allergen

Raphaela Rid^{a,1}, Kamil Önder^{a,b,1}, Susan MacDonald^c, Roland Lang^b, Thomas Hawranek^b,
Christof Ebner^d, Wolfgang Hemmer^e, Klaus Richter^a, Birgit Simon-Nobbe^a, Michael Breitenbach^{a,*}

^a Department of Cell Biology, University of Salzburg, Hellbrunnerstraße 34, 5020 Salzburg, Austria

^b Division of Molecular Dermatology, Department of Dermatology, St. Johannis-Spital, Müllner Hauptstraße 48, 5020 Salzburg, Austria

^c Department of Medicine, Division of Clinical Immunology, Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224, USA

^d Allergie-Ambulatorium Reumannplatz, Reumannplatz 17, 1100 Vienna, Austria

^e Floridsdorfer Allergiezentrum, Franz-Jonas-Platz 8, 1210 Vienna, Austria

ARTICLE INFO

Article history:

Received 21 June 2009

Accepted 26 July 2009

Available online xxx

Keywords:

Allergy

Alternaria alternata

Ascomycete

Fungi

Mold

Recombinant protein

TCTP

ABSTRACT

Defining more comprehensively the allergen repertoire of the ascomycete *Alternaria alternata* is undoubtedly of immense medical significance since this mold represents one of the most important, worldwide occurring fungal species responsible for IgE-mediated hypersensitivity reactions ranging from rhinitis and ocular symptoms to severe involvement of the lower respiratory tract including asthma with its life-threatening complications. Performing a hybridization screening of an excised *A. alternata* cDNA library with a radioactively labeled *Cladosporium herbarum* TCTP probe, we were able to identify, clone and purify the respective *A. alternata* homologue of TCTP which again represents a multifunctional protein that has been evolutionarily conserved from unicellular eukaryotes like yeasts to humans and appears, summarizing current literature, to be involved in housekeeping processes such as cell growth as well as cell-cycle progression, the protection of cells against various stress conditions including for instance apoptosis, and in higher organisms even in the allergic response. In this context, our present study characterizes recombinant *A. alternata* TCTP as a novel minor allergen candidate that displays a prevalence of IgE reactivity of approximately 4% and interestingly shares common, cross-reactive IgE epitopes with its *C. herbarum* and human counterparts as determined via Western blotting and *in vitro* inhibition approaches.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Out of the enormous amount of known fungal species that again represent eukaryotic, non-chlorophyllous, cell-wall surrounded, principally spore-bearing organisms with a rather heterogenous phenotype ranging from unicellular to dimorphic or filamentous appearance, exist as either symbionts, saprophytes or parasites of both animals and plants under virtually all ecological conditions and typically produce teleomorph as well as anamorph stages which possess the capability to propagate independently from each other and are morphologically clearly distinguishable, *bona fide* mold allergens responsible for an induction of type I hypersensitivities within susceptible individuals have been isolated from

only 23 genera so far (Bush and Prochnau, 2004; Crameri et al., 2006; Horner et al., 1995; Kurup and Banerjee, 2000; Kurup et al., 2002; Simon-Nobbe et al., 2008). Since airborne mold spores are ubiquitously present in concentrations ranging from 230 to 1×10^6 particles per m^3 depending on climatic parameters such as temperature, wind, moisture and for this reason nutrient availability, thereby exceeding the average tree or grass pollen intensity approximately 100–1000 times, about 6–24% of the general population, nearly 44% among atopic individuals and even up to 80% within asthmatics are affected by at least one manifestation of IgE-mediated fungal allergy, a well-known medical affliction (Crameri et al., 2006; Frew, 2004; Horner et al., 1995; Kurup et al., 2000; Simon-Nobbe et al., 2008). In contrast to the majority of inhaled particles that possess a diameter of more than $10 \mu m$ such as pollen or clusters of small *Aspergillus conidia* and are deposited in the nasopharynx where they provoke the typical nasal or ocular symptoms usually referred to as hay fever, fungal spores on average are merely about 2–3 μm in size, can penetrate the terminal airways in the case of inhalation and are thus associated with both upper and lower respiratory symptoms (Frew, 2004; Horner et al., 1995; Kurup and Banerjee, 2000; Kurup et al., 2000, 2002). Whereas the majority of fungi commonly considered to be allergenic such as *Alternaria* and *Cladosporium* display a seasonal

Abbreviations: AA, amino acid; *A. alternata*, *Alternaria alternata*; AaTCTP, *A. alternata* TCTP; CD, circular dichroism; *C. herbarum*, *Cladosporium herbarum*; ClaTCTP, *C. herbarum* TCTP; 6×HIS, hexahistidine-tag; hTCTP, human TCTP; IPTG, isopropyl-β-D-thiogalactopyranoside; NHS, normal human serum; RAST, radioallergosorbent testing; rnf, recombinant non-fusion; SC, synthetic complete medium; TCTP, translationally controlled tumor protein; TEV, tobacco etch virus.

* Corresponding author. Tel.: +43 662 8044 5787 fax: +43 662 8044 144.

E-mail address: Michael.Breitenbach@sbg.ac.at (M. Breitenbach).

¹ These authors contributed equally to the work.

spore release pattern that peaks during summer and fall months and has the tendency to decrease with cooler temperatures (Burge, 2002; Horner et al., 1995; Sanchez and Bush, 2001), so-called indoor fungi on the other hand are a mixture of molds growing in indoor locations such as *Aspergillus* or *Penicillium* and those entering from outdoors, therefore vary substantially in their presence and amount depending on humidity, ventilation, the availability of biologically degradable material and the presence of pets as well as plants and are in combination with allergens from house dust mites, cockroaches and animal dander an important cause of indoor sensitization that result in omnipresent mold exposure situations and explain why most of the fungi-sensitive patients rather suffer from perennial symptoms which can contribute to both the chronicity and severity of asthma (Cramer et al., 2006; Jacob et al., 2002; Kurup and Banerjee, 2000; Portnoy et al., 2004; Terr, 2004).

Screening of fungal cDNA libraries constructed in the bacteriophage λ with sera of mold-sensitized patients or the cloning of allergens by phage-display that allows repeated rounds of enrichment by bio-panning has enabled a steady progress in the characterization of *A. alternata*, *Cladosporium herbarum* and *Aspergillus fumigatus* allergens within the last two decades (Achatz et al., 1995, 1996; Breitenbach and Simon-Nobbe, 2002; Cramer et al., 1996). In this context, we here present the identification of *A. alternata* TCTP as a novel, minor ascomycete allergen which we (i) isolated via hybridization screening from an *in vivo* excised phage library, (ii) produced as a pure recombinant non-fusion protein and (iii) tested for a selection of molecular features and immunological properties including its *in vitro* clinical relevance. We finally indicate that *A. alternata* TCTP, following its officially recognized approval by the respective health department, could be included in a minimal testing kit upon the suspicion of fungal allergy, because a usage of highly cross-reactive allergens instead of numerous homologues of the same protein class could simplify and in this way perhaps even reduce expenses of allergy diagnosis.

2. Materials and methods

2.1. Reagents, sera, primers and plastic ware

Eppendorf tubes and petri dishes were bought from Greiner Holding AG, Kremsmünster, Austria, chemicals for buffers, solutions or media from Sigma-Aldrich, Taufkirchen, Germany, Roth, Karlsruhe, Germany, or Applichem GmbH, Darmstadt, Germany via GenXpress Service/Vertrieb GmbH, Maria Wörth, Austria. Cloning and restriction enzymes were purchased from Fermentas Life Sciences GmbH, St. Leon-Rot, Germany, Finnzymes, Espoo, Finland, or New England Biolaboratories, Ipswich, MA, USA, sequencing reagents from Applied Biosystems, Foster City, CA, USA. Primers were synthesized by MWG-Biotech AG, Ebersberg, Germany. Radioactively labeled anti-human IgE was obtained from MedPro, Vienna, Austria. Human sera were supplied by three different Austrian allergy clinics, the Department of Dermatology at the St. Johanns-Spital, Salzburg, Austria, the Floridsdorfer Allergiezentrum, Vienna, Austria, and the Allergie-Ambulatorium Reumannplatz, Vienna, Austria, and stored at -20°C . Samples were taken from a group of patients with a typical case history of immediate hypersensitivity reactions to *Alternaria alternata* (and eventually *C. herbarum*), a positive skin prick test response to commercial *A. alternata* mold extract or mold mix and a corresponding RAST class greater than 3. Sera from a non-allergic healthy subject (NHS) as well as one birch-allergic individual non-allergic to fungal allergens were used as negative control in IgE immunoblots.

2.2. Database searches, computer-assisted analyses and protein modeling

Nucleotide and protein sequences used within this study were retrieved from the GenBank and Pubmed databases at the National Center for Biotechnology Information NCBI homepage available via www.ncbi.nlm.nih.gov/, the Universal Protein Resource UniProt (www.uniprot.org/) or the Biobase server (www.biobase-international.com/). Additional features such as detailed sequence comparisons, homology searches, the calculation of protein parameters or secondary structure predictions were mainly conducted through the basic local alignment search tool BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), the ExPASy Proteomics Server (<http://www.expasy.ch/>), the PSIPRED database at bioinf.cs.ucl.ac.uk/psipred/ accessible via the BioInfoBank Metaserver (meta.bioinfo.pl/) or the SWISS-PROT protein knowledgebase (<http://expasy.org/sprot/>). Multiple sequence alignments were obtained using the MultAlin program (bioinfo.genotoul.fr/multalin/multalin.html) and respective results further processed utilizing Boxshade (www.ch.embnet.org/software/BOX_form.html) version 3.21. The three-dimensional model of *A. alternata* TCTP was constructed using the online available program 3D JIGSAW accessible via bmm.cancerresearchuk.org/~3djigsaw/, a software which splits the query sequence into small elements and searches for homologous templates in various databases such as for instance PFAM (pfam.sanger.ac.uk/) and the RCSB Protein Data Bank PDB at www.rcsb.org/. The resultant structure was analyzed by using RASWIN (version 2.6) or FATCAT (flexible structure alignment by chaining aligned fragment pairs allowing twists, fatcat.burnham.org/). Putative linear B-lymphocyte epitopes were calculated by utilizing BepiPred 1.0 (www.cbs.dtu.dk/services/BepiPred/) that again employs a combination of hidden Markov models as well as propensity scale methods and marked in the three-dimensional *A. alternata* structure, respectively, applying Swissmodel (swissmodel.expasy.org/) and UCSF-Chimera downloaded from www.cgl.ucsf.edu/chimera/. ClustalW (www.ebi.ac.uk/clustalw/) was considered to draw a phylogenetic tree by comparing *C. herbarum* and *A. alternata* TCTP with homologous protein sequences from a selection of other eukaryotic organisms.

2.3. Cultivation of *A. alternata* and preparation of a crude fungal protein extract

The ascomycete *A. alternata* (strain collection number 08-0203), obtained from the Institute of Applied Microbiology (IAM), University of Agricultural Sciences, Vienna, Austria, was grown in SC-medium in flat 94 mm petri dishes and incubated at 23°C until a closed velvety fungal mat containing both vegetative hyphae as well as conidiophores developed that was after about 5 days of growth finally removed from the surface of the petri dish, put into a mortar filled with liquid nitrogen and manually ground with a pre-chilled pestle (Achatz et al., 1995, 1996; Breitenbach and Simon-Nobbe, 2002; Schneider et al., 2006). The resultant fine powder was extracted overnight in buffer containing 10 mM sodium phosphate (pH 7.5), 2 mM EDTA, 10 μl protease inhibitors (0.2 mg/ml aprotinin, 70 $\mu\text{g/ml}$ pepstatin, 10 mg/ml bacitracin, 0.1 mg/ml antipain, 0.2 mg/ml leupeptin, or alternatively, instead one CompleteTM protease cocktail tablet, Roche Diagnostics GmbH, Mannheim, Germany per 100 ml buffer) under constant agitation at 4°C (Achatz et al., 1995, 1996). After centrifugation at $5000 \times g$ for 40 min at 4°C , the supernatant was collected and the protein content within measured using a Coomassie Protein Assay Reagent Kit (PIERCE, Rockford, IL, USA) according to Bradford (Bradford, 1976) with BSA as a standard. Protein aliquots were dia-

lyzed against water, lyophilized and stored at -20°C . Crude fungal protein extracts were analyzed by denaturing gel electrophoresis according to Laemmli (Laemmli, 1970) and visualized by either dyeing with Coomassie Brilliant Blue R-250 or silver staining with the SilverXpress kit (Invitrogen, Carlsbad, CA, USA).

2.4. Isolation of *A. alternata* genomic DNA

A. alternata genomic DNA was isolated as described previously (Al-Samarrai and Schmid, 2000). In brief, 30 mg of mycelium were ground to a fine powder in liquid nitrogen and resuspended in 500 μl lysis buffer (40 mM Tris–acetate, pH 7.8, 20 mM sodium acetate, 1 mM EDTA, 1% SDS) until the viscosity of the suspension was obviously reduced. Following the addition of 2 μl RNase A (10 mg/ml) and a 15 min incubation at 37°C , 165 μl 5 M NaCl were supplemented to facilitate the precipitation of most polysaccharides and cellular as well as protein debris. The suspension was centrifuged at $12,000 \times g$ for 20 min at 4°C . The obtained supernatant was transferred to a fresh tube, PCI-extracted according to a standard procedure, the DNA in the aqueous supernatant precipitated with two volumes of 95% ethanol, washed with 70% ethanol, dissolved in 50 μl 1 $\times$ TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.8) and finally stored at -20°C .

2.5. Southern blot analysis

30 μg of genomic *A. alternata* DNA was digested with EcoRI and BamHI at 37°C for 12 h. The resultant fragments were concentrated by isopropanol precipitation, the dried pellet dissolved in 30 μl 1 $\times$ TE buffer, fractionated by 0.7% agarose gel electrophoresis in 1 $\times$ TAE at 50–80 V for 6 h and ultimately transferred in 10 $\times$ SSPE buffer (1.5 M NaCl, 100 mM sodium phosphate, pH 7.0, 10 mM EDTA) to a GENESCREENTM nylon membrane (Perkin Elmer, Boston, MA, USA) overnight via the capillary effect. The next day, the membrane was irradiated at 254 nm with 1200 mJ to crosslink the DNA to the membrane (Stratalinker, Stratagene, Agilent Technologies, Santa Clara, CA, USA). A radioactively labeled DNA probe was prepared by PCR amplification of the corresponding *C. herbarum* TCTP sequence (Rid et al., 2008) via 5'-CGGGATCCATGCTCATCTACAAGGA-3' ($T_M = 55.6^{\circ}\text{C}$) and 5'-AAGGCCTTACACCTTGGTGGACTT-3' ($T_M = 54.9^{\circ}\text{C}$). Following gel purification (Wizard[®] SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA), 25 ng DNA were randomly labeled with (α -³²P)dCTP according to the Primer-a-Gene[®] Labeling System (Promega, Madison, WI, USA) and denatured at 95°C for 3 min prior to the addition of 20 mM EDTA. The hybridization was carried out in roller bottles at 55°C overnight in buffer containing 6 $\times$ SSPE, 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA, 0.5% SDS as well as 5 $\times$ Denhardt's reagent (0.1% BSA, 0.1% polyvinylpyrrolidone, 1% Ficoll), followed by three consecutive washing steps, drying of the membrane and exposition to either an X-ray (KODAKTM, Rochester, NY, USA) or a high performance autoradiography film (HyperfilmTM, GE Healthcare, Uppsala, Sweden) for 6 days at -70°C .

2.6. Preparation of an *A. alternata* cDNA library in the λ -ZAP system

An *A. alternata* cDNA library in the λ -ZAP II System (Stratagene, La Jolla, CA, USA) was constructed according to the standard Stratagene protocol as described previously (Achatz et al., 1995, 1996; Breitenbach and Simon-Nobbe, 2002). In short, mycelium was harvested and crushed under liquid nitrogen, the pulverized mold powder resuspended in 12 ml/g guanidinium isothiocyanate containing 1% β -mercaptoethanol, homogenized using an Ultra-TurraxTM apparatus (IKA-Werke, Staufen, Germany) and total RNA finally extracted once with water-saturated phenol/chloroform and

ultimately with chloroform/isoamylalcohol after a digestion with proteinase K. Following the precipitation of the aqueous phase with an equal amount of isopropanol at -20°C , polyA⁺ RNA was next isolated from the total RNA pool via oligo-(dT) cellulose chromatography and reverse transcribed into cDNA which was next purified over Sepharose CL-4B columns (GE Healthcare, Uppsala, Sweden) and ligated directionally into the Uni-ZAP[®] XR phage vector. Obtained phages were in a last step subjected to a standard *in vivo* excision protocol by co-infection with an ExAssist helper phage to release their cDNA inserts from the λ backbone into pBluescript SK (Stratagene, La Jolla, CA, USA).

2.7. Isolation of an *A. alternata* TCTP homologue via hybridization screening

About 1.9×10^5 *Escherichia coli* SOLRTM cells carrying the excised pBluescript SK-plasmid constructs were grown overnight on LB-amp plates (diluted to a density of 5000 clones per large petri dish) and nitrocellulose filters (PROTRAN[®], 0.45 μm pore size, Schleicher and Schüll, Dassel, Germany) layered on top of them in order to lift colonies. DNA was denatured for 5 min with 1.5 M NaCl/0.5 M NaOH, neutralized with 1 M Tris–HCl (pH 7.4)/1.5 M NaCl for further 5 min and after rinsing the filters with 2 $\times$ SSPE crosslinked to the membrane. Prehybridization in buffer containing 0.1 M sodium phosphate, 850 mM NaCl, 2.5 mM EDTA, 0.1% SDS, 10 $\times$ Denhardt's solution and 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA was performed for 1 h at 60°C prior to overnight hybridization with the randomly (α -³²P)dCTP labeled *C. herbarum* TCTP fragment. High stringency washes were conducted with prewarmed 4 $\times$ SSPE, 2 $\times$ SSPE and 1 $\times$ SSPE all containing 0.1% SDS for 30 min each before the filters were air-dried and exposed to HyperfilmTM. Clones giving positive signals were picked and their plasmid DNA isolated with the GFXTM Micro Plasmid Preparation Kit (GE Healthcare, Uppsala, Sweden). The full-length sequence of *A. alternata* TCTP candidate molecules was finally determined on both strands by using vector-specific T₃ and T₇ primers (5'-AATTAACCTCACTAAAGGG-3', $T_M = 48.9^{\circ}\text{C}$, 5'-GTAATACG-ACTCACTATAGGGC-3', $T_M = 49.6^{\circ}\text{C}$) as well as a variety of internal oligonucleotides via the ABI PRISMTM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISMTM 310 Genetic Analyzer (Applied Biosystems, Vienna, Austria).

2.8. Subcloning of *A. alternata* TCTP into pHIS parallel 2

The open reading frame encoding the putative new mold allergen was amplified from the original pBluescript SK-clones using gene-specific oligodesoxynucleotides, i.e., a forward primer with a BamHI (underlined) restriction site in front of the start codon (5'-CGGGATCCATGCTCATCTACAAGGA-3', $T_M = 64.6^{\circ}\text{C}$) and a 3' reverse primer introducing an EcoRI (underlined) restriction site (5'-GGAATTCCTAGACCTTCATCTCC-TCGA-3', $T_M = 63.4^{\circ}\text{C}$), as well as a proofreading DNA polymerase. Using regular molecular biology techniques, the 510 nucleotide amplification product was subcloned into the appropriately restricted, dephosphorylated vector pHIS parallel 2 which encodes an N-terminal hexahistidine-tag for simplified protein purification approaches, followed by spacer region with a TEV protease cleavage site and a common polylinker sequence generated by Sheffield et al., referred to as 6 $\times$ HIS-AaTCTP (Rid et al., 2008; Sheffield et al., 1999). Following a transformation into the *E. coli* strain BL21(DE3), the constructs harbouring inserts of expected size were sequenced according to a Dye Terminator Cycle Sequencing protocol using vector-specific primers (pHIS forward: 5'-CCATCACGATTACGATATCCC-3', $T_M = 61.4^{\circ}\text{C}$, reverse: 5'-CAACTCAGCTTCCTT-TCTG-3', $T_M = 58.3^{\circ}\text{C}$) in order to ensure the authenticity of the cloned nucleotide sequence.

2.9. Purification of *A. alternata* TCTP via Ni^{2+} affinity chromatography

E. coli BL21(DE3) cells transformed with 6×HIS-AaTCTP were grown in LB medium supplemented with 100 mg/l ampicillin at 37° until an OD₆₀₀ of 0.8 was reached. LacZ-promotor mediated expression of target genes was induced by the addition of 0.5 mM IPTG before cells were further grown for 20 h at 16 °C. Cells were harvested by centrifugation and the resultant pellet finally resuspended in 1/50 volume of Starting Buffer (50 mM Na₂HPO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole). *A. alternata* TCTP was isolated from the bacterial lysate via addition of 1 mg/ml lysozyme, 10 µg/ml RNase A as well as 5 µg/ml DNase I and three consecutive cycles of freezing and thawing prior to shearing the genomic DNA by ultrasonication, a removal of the insoluble protein fraction by spinning at 10,000 × g for 30 min and lastly a filtration through a 0.45 µm low-protein binding Millex-HV filter (Millipore Corporation, Bedford, MA, USA). For the purification of *A. alternata* TCTP and its *C. herbarum* and human homologues via immobilized metal affinity chromatography (Chelating Sepharose® Fast Flow, GE Healthcare, Uppsala, Sweden) under native conditions, soluble bacterial lysates were directly loaded on regenerated columns charged with 0.2 volumes 0.1 M NiSO₄ as reported in detail elsewhere (Rid et al., 2008). A competitive gradient elution was carried out via the step-wise increase from 100 to 500 mM imidazole at a constant pH under gravity flow. After dialysis against 20 mM phosphate buffer (pH 7.4), the elution product was then used for cleaving its 6×HIS fusion tag utilizing commercially available, recombinant Tobacco etch virus TEV protease (AcTEV™, 10 U/µl, Invitrogen, Karlsruhe, Germany). Digestion was allowed to proceed at 29 °C for 4 h and at 4 °C overnight with 1 U enzyme per 6 µg protein, followed by a second one-step passage over the metal affinity column in the course of which the native rnf protein was collected in the flow through fractions, referred to as rnf-AaTCTP, whereas the AcTEV™ protease was removed from the reaction by binding to the Chelating Sepharose® material via a genetically engineered polyhistidine tag at its own N-terminus (Kapust et al., 2001). Subsequent to measuring protein concentrations using a commercial kit (Bio-Rad Laboratories, Hercules, CA, USA), homogeneity of the eluted fractions was again monitored by SDS-PAGE and silver staining. Fractions containing protein with an apparent purity of >95% were pooled, dialyzed against water and aliquoted.

2.10. IgE immunoblots and inhibition immunoblots

Immunoblot analyses with 1.5 µg rnf-AaTCTP per reaction were performed as described previously (Rid et al., 2008; Schneider et al., 2006). Membrane strips were for that purpose stained with 0.1% Ponceau-Red in 1% acetic acid, blocked with Gold Buffer (40 mM Na₂HPO₄, 7 mM NaH₂PO₄, 0.5% BSA, 0.5% Tween-20) for 60 min and probed with different patients' sera diluted 1:10 overnight at 4 °C. Cursorily, 16 sera of individuals with a case history of immediate type I hypersensitivity to *A. alternata* as well as a positive skin prick test response to commercial *A. alternata* extract or mold mix as well as further nine samples preselected for positive IgE reactivity towards human and *C. herbarum* TCTP were investigated in the course of these preliminary studies. Binding of patients' IgE antibodies to *A. alternata* TCTP was detected by scanning imaging plates (Fujifilm BAS Cassette 2325) with a Fujifilm BAS-1800 II instrument using the corresponding BAS-Reader software (version 2) for Windows (Raytest, Straubenhardt, Germany) after 2 days of exposure. For a detailed testing of the competitive IgE binding capacity to (i) *C. herbarum* TCTP and (ii) *A. alternata* TCTP by inhibition analysis, selected TCTP-reactive sera were preincubated overnight with (i) 100 µg rnf-hTCTP or 100 µg rnf-AaTCTP as well as (ii) 100 µg rnf-hTCTP and 100 µg rnf-ClatCTP, respectively, and then used to probe

rnf-ClatCTP and rnf-AaTCTP PVDF membrane strips as described above. Conversely, serum samples preincubated with 100 µg rnf-ClatCTP and 100 µg rnf-AaTCTP were utilized to analyze membrane strips blotted with rnf-hTCTP.

2.11. Circular dichroism (CD) spectroscopy measurements

Circular dichroic spectra of 6×HIS-AaTCTP and rnf-AaTCTP (working protein concentration: 0.1 µg/µl) in the range between 190 and 260 nm were recorded in 10 mM sodium phosphate buffer (pH 7.4) with a J-810 spectropolarimeter (JASCO Inc., Easton, MD, USA) fitted with a Neslab RTE-111 M temperature control system (Thermo Neslab Inc., Newington, NH, USA) in continuous scanning mode using a 1.0 mm path length quartz cuvette, a 1 nm resolution, a 100 mdeg sensitivity, a 100 nm/min scanning speed and a 1 nm bandwidth. The baseline obtained with pure buffer in the absence of protein was subtracted from the sample spectrum. Data were expressed as mean residue molar ellipticity (Θ) taking protein concentration, molecular weight and the number of peptide bonds into account (Rid et al., 2008).

3. Results

3.1. Identification, isolation and characterization of *A. alternata* TCTP

Because the to date identified and in part already comprehensively characterized recombinant *A. alternata* allergens are not sufficient to explain the heterogenous, rather complex patterns of IgE reactivity observed in *A. alternata* allergic individuals via one- or two-dimensional *in vitro* immunoblot testings using the corresponding crude fungal protein extract, our research group has, based on the assumption that hence several hypersensitivity reaction eliciting molecules were still unknown, put much effort into the molecular cloning and description of novel fungal allergens in the last years (Achatz et al., 1995, 1996; Breitenbach and Simon-Nobbe, 2002; Rid et al., 2008; Schneider et al., 2006; Simon-Nobbe et al., 2000). In order to provide evidence for the existence of a so far unknown *A. alternata* TCTP protein (i) whose *C. herbarum* homologue has in 2008 been reported to represent a minor ascomycete allergen (Rid et al., 2008) and which (ii) we theoretically hypothesized to subsist with significant likelihood due to its extreme degree of evolutionary conservation and because of its involvement in essential intracellular housekeeping functions (Bommer and Thiele, 2004), we initially performed a preliminary Southern Blot using BamHI and EcoRI digested fungal genomic DNA separated on a 0.7% agarose gel and a radioactively (α-³²P)-labeled PCR product prepared from the available *C. herbarum* TCTP sequence information, an approach that indeed proved to be successful (data not shown). Secondly, we focused our attention on the concrete isolation of this particular *A. alternata* TCTP molecule by screening the respective *in vivo* excised mold cDNA expression library prepared in the bacteriophage λ from both fungal mycelia as well as spores applying the same radioactively labeled *C. herbarum* TCTP DNA probe and definitely resulted in the isolation of two full-length and five incomplete, N-terminally shortened *A. alternata* TCTP clones as determined by sequencing the corresponding phagemid DNAs on both strands using vector-derived oligonucleotides and their further analysis via bioinformatical studies.

As illustrated in Fig. 1, *A. alternata* TCTP contains an open reading frame spanning 510 nucleotides that starts with the first ATG initiation codon at nucleotide position 104, ends with a TAA termination triplet at position 614, is flanked by a 103 bp 5'-UTR and a complete 152 bp 3'-UTR followed by a poly(A) tail, and possesses a calculated molecular weight of 18.8 kDa as well as an isoelectric

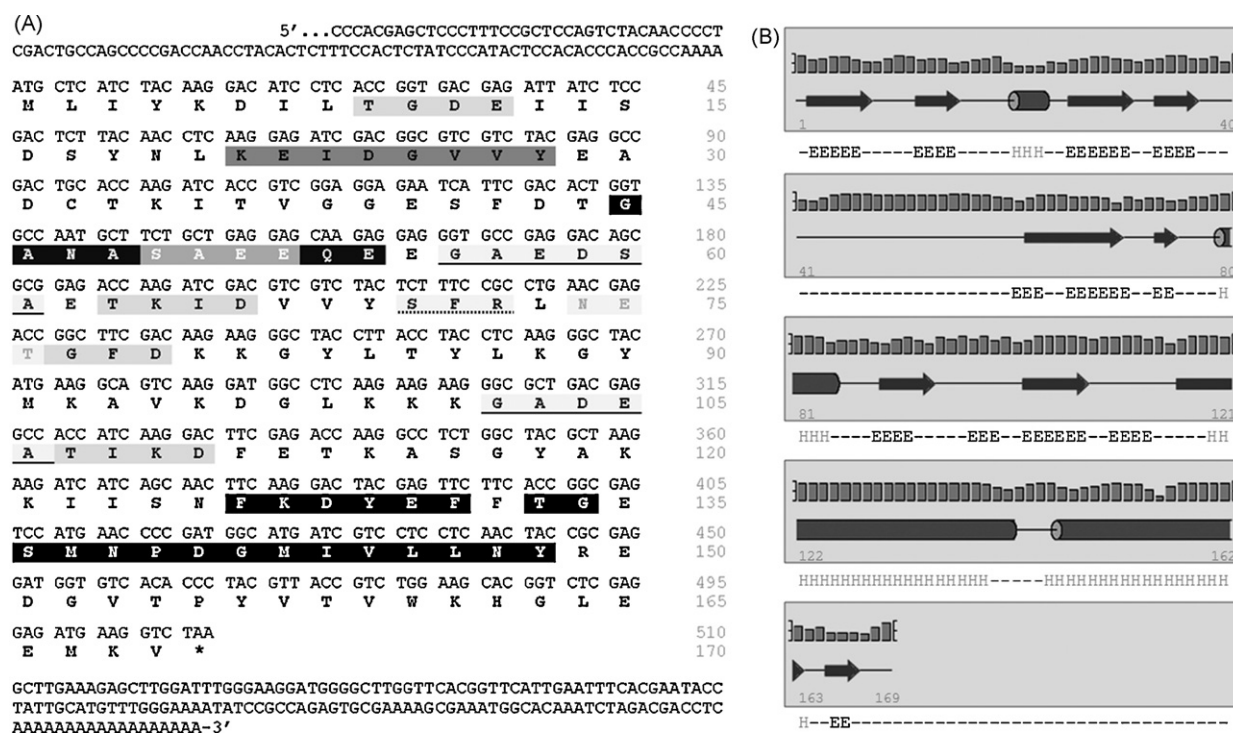


Fig. 1. *A. alternata* TCTP sequence. (A) Nucleotide and deduced amino acid sequence of the isolated full-length *A. alternata* TCTP (numbers on the right side denote the actual positions). The highly conserved TCTP signature 1 and 2 sequences are highlighted with black boxes. Transcription is initiated at the first ATG start codon available in the sequence. The translation termination codon TAA is marked by an asterisk. The in part overlapping sites for putative casein kinase II phosphorylation (marked in light grey), protein kinase C phosphorylation (underlined with dots), tyrosine kinase phosphorylation (shown in dark grey), N-glycosylation (grey letters) and myristoylation (underlined with black contour) are indicated as specified in brackets. The complete cDNA sequence was submitted to the National Center for Biotechnology Information (NCBI) under accession number 828232. (B) Computed secondary structure elements of *A. alternata* TCTP predicted via Psipred and Metaserver. Lines represent coiled regions, grey arrows indicate β -strands (E) and black cylinders symbolize α -helices (H). $\uparrow$ and $\downarrow$ represent the confidence interval (0 = low, 9 = high degree of probability), respectively.

point (pI) of about 4.59 as determined through the Expert Protein Analysis System. Parenthetically, a PCR amplification of TCTP from genomic *A. alternata* DNA isolated according to Al-Samarrai and Schmid (2000) using gene-specific subcloning primers and the subsequent sequencing of both sense- and antisense strands has revealed no difference in the nucleotide composition to the respective cDNA (data not shown), thereby indicating the absence of any introns. The complete cDNA as well as protein sequence of *A. alternata* TCTP was next deposited in the National Center for Biotechnology Information GenBank NCBI database under accession number 828232. In more detail, the *A. alternata* TCTP protein is composed of 169 amino acids, namely 11 alanines (6.5%), 2 arginines (1.2%), 6 asparagines (3.6%), 19 lysines (11.2%), 15 aspartic acid residues (8.9%), 1 cysteine (0.6%), 1 glutamine (0.6%), 18 glutamic acid residues (10.7%), 16 glycines (9.5%), 1 histidine (0.6%), 11 valines (6.5%), 2 prolines (1.2%), 1 tryptophan (0.6%), 11 isoleucines (6.5%), 12 threonines (7.1%), 7 phenylalanines (4.1%), 5 methionines (3.0%), 9 serines (5.3%) and 11 tyrosine residues (6.5%), or in other words contains altogether a total number of 33 negatively (D, E) and of 21 positively charged (R, K) amino acids, and accordingly has a calculated extinction coefficient of $21,890 \text{ U} \times \text{M}^{-1} \times \text{cm}^{-1}$ as evaluated using ProtParam, a bioinformatic tool allowing the computation of several physical and chemical parameters for a user-entered sequence (Bairoch et al., 2005; Gasteiger et al., 2001). Analogously to its *C. herbarum* counterpart, *A. alternata* TCTP does not demonstrate any obvious N-terminal leader sequences, nuclear localization signals or hydrophobic transmembrane anchors as determined using the SignalP 3.0 server at <http://www.cbs.dtu.dk/services/SignalP/output.html> and has been reliably predicted to be a cytoplasmic molecule with overall hydrophilic properties (aliphatic index: 73.85, grand average of hydrophobicity: -0.483) via the PSORT analysis

(<http://www.psорт.ims.u-tokyo.ac.jp/>). Its sequence-based three-dimension model computed with the Prediction Server PSIPRED as well as the comparative protein modeling program 3D JIGSAW (i) exhibits considerable similarity to the *Schizosaccharomyces pombe* orthologue whose overall architecture, recently determined by nuclear magnetic resonance NMR spectroscopy (PDB codes: 1H6Q or 1H7Y), unexpectedly displays a substantial relationship to the human protein Mss4, a guanine nucleotide-free chaperone of the Rab protein implicated in vesicle transport, intracellular trafficking and thus the secretory pathway (Thaw et al., 2001), and (ii) furthermore shares a noticeable conservation concerning both the primary sequence and three-dimensional structure with the closely related *C. herbarum* TCTP (64% homology, 80% similarity) as well as homologues from several other species including for instance *Saccharomyces cerevisiae*, *S. pombe*, *Mus musculus* and *Homo sapiens* (also known as histamine-releasing factor, PDB code: 1Y21) TCTP (Figs. 1–3A) what together with its ubiquitous expression underscores the central role TCTP is believed to play in the physiology of eukaryotic organisms (Baxter et al., 2000; Bommer and Thiele, 2004; Feng et al., 2007). Fig. 3A besides shows that the protein consists of an α -helical region to which microtubule- and Ca^{2+} -binding sites have been mapped, a β -stranded core-domain where the majority of invariant residues are clustered, signifying that this site is extremely important for molecular interactions, and a flexible loop that tolerates minor insertions as well as deletions (Bommer and Thiele, 2004; Gachet et al., 1999; Thaw et al., 2001). The neighbour-joining phylogram view in Fig. 2B once more exemplifies that ascomycete TCTPs are undeniably directly interrelated and clustered in a separate branch together with *S. cerevisiae* and *S. pombe* TCTP, implying an early origin for the TCTP orthologue in eukaryotic evolution approximately 1×10^9 years ago as previously estimated by Venugopal (2005) and Hinojosa-

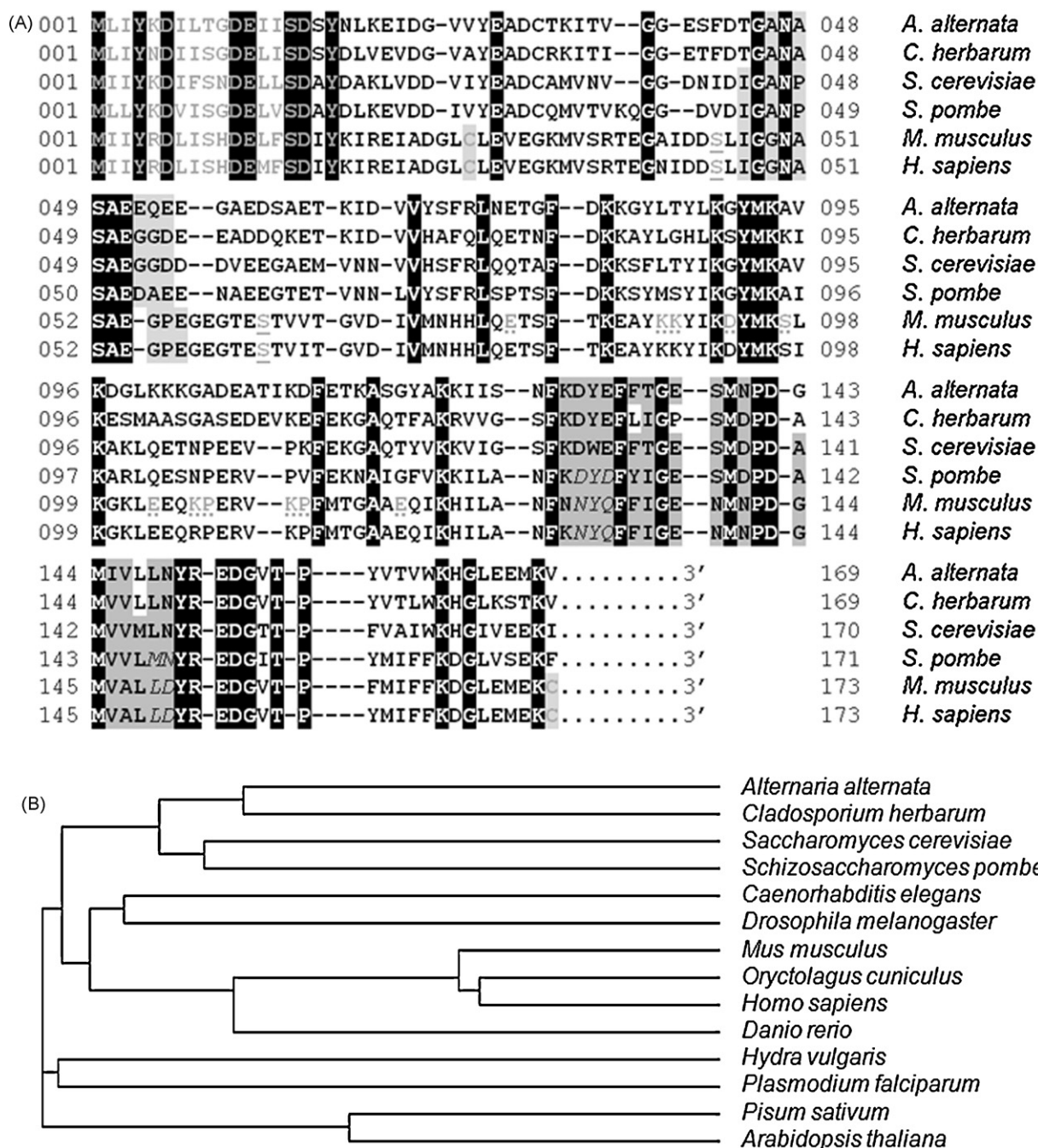


Fig. 2. Multiple sequence alignment. (A) ClustalW alignment of several AA sequences of the conserved TCTP family of proteins, showing the high degree of homology between phylogenetically distinct members, but no primary sequence homology to any other protein family. The N-terminal methionine is included as a conserved residue since it is present in the human native protein. Identical AA are highlighted in black. The TCTP signature 1 and 2 sequences listed in Prosite are marked with light grey boxes, the newly discovered conserved domain from position 1 to 16 in grey letters. Residues of the mouse sequence that match AA within part of the tubulin-binding region of human MAP-1B are underlined with dots. Serine residues (only conserved in higher eukaryotes) in the mouse TCTP that are phosphorylated by mitotic Plk kinase are underlined. Human and mouse TCTP contain 2 conserved cysteine residues (shaded in grey), but it has not been studied whether these residues are actually engaged in disulfide bond formation. The residues involved in Ca²⁺ binding are given in italics. (B) The ClustalW program was used to construct a neighbour-joining phylogenetic tree by comparing the *C. herbarum* and *A. alternata* TCTP protein sequence with those of other organisms including yeast, worm, fly, mouse, rabbit, human and plant.

Moya et al. (2008). Consistent with the characteristic features of TCTP and comparable to the minor constrictions already described for *C. herbarum* TCTP (Rid et al., 2008), supplementary pattern and profile searches in the InterPro and PROSITE database revealed that *A. alternata* TCTP (Figs. 1A and 2A) undoubtedly possesses a clearly defined TCTP signature 1 sequence at positions 45–54 [GA]-[GAS]-N-[PAK]-S-[GTA]-E-[GDEV]-[PAGEQV]-[DEQGAV] and a typical TCTP signature 2 region (AA 128–142) with the consensus [FLIV]-X₍₄₎-[FLVH]-[FY]-[MIVCT]-G-E-X₍₄₎-[DENP]-[GAST]-

x-[LIVM]-[GAVI]-X₍₃₎-[FYWQ] that is generally found within all of the to date known TCTPs except the *Hydra vulgaris* (Yan et al., 2000) and *Schistosoma mansoni* homologues (Rao et al., 2002), an observation that could explain the lack of cross-reactivity between parasitic and metazoan TCTPs. The *A. alternata* TCTP protein furthermore harbours several theoretical phosphorylation sites (Fig. 1A), namely 2 (AA 70–72, AA 107–109) for protein kinase C phosphorylation, 5 for casein kinase II (AA 9–12, 49–52, 63–66, 76–79, 107–110) and 1 (AA 21–28) for tyrosin kinase as determined

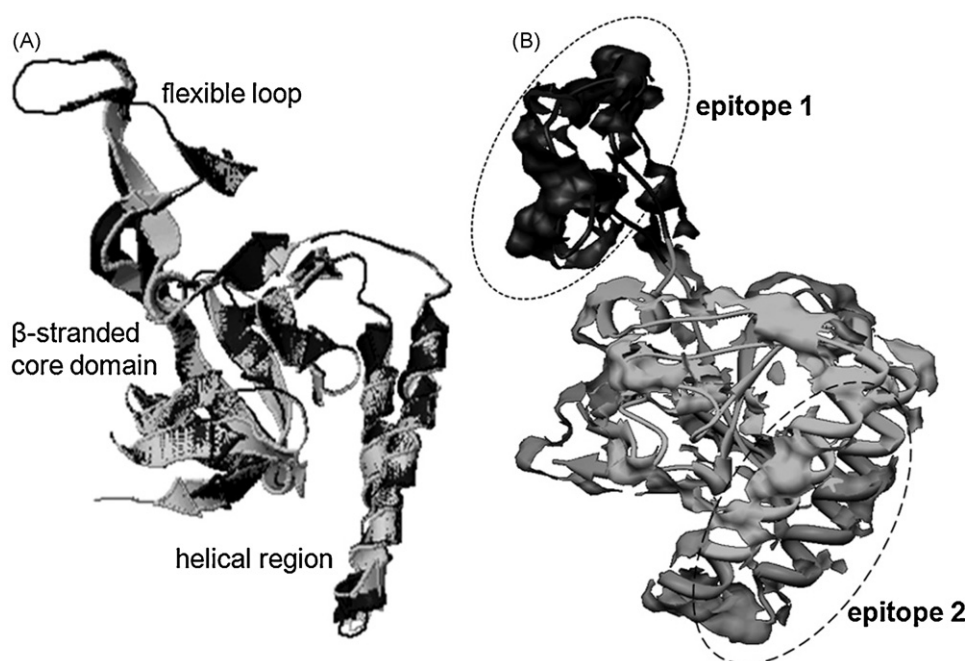


Fig. 3. Structural modeling of *A. alternata* TCTP. (A) The three-dimensional structure of *A. alternata* TCTP (black) that was predicted based on the recently published three-dimensional model of *S. pombe* TCTP and the solution structure of human TCTP determined by NMR spectroscopy that is available in the RCSB database (www.rcsb.org/pdb, pdb code: 2HR9) was superimposed over the *C. herbarum* TCTP molecule (grey). (B) Illustration of the two putative linear B-lymphocyte epitopes of *A. alternata* TCTP. Black: *A. alternata* TCTP epitope 1 (ITVGESFDTGANASAEQEEGAEDSAET), dark grey: *A. alternata* TCTP epitope 2 (GLKKKGADATIKDFETKASGY).

utilizing the NetPhos 2 Program provided by the Technical University of Denmark (<http://www.cbs.dtu.dk/services/NetPhos/>), 2 N-glycosylation patterns (AA 47–50, 74–77) as analyzed via the NetNGlyc 1 Server at <http://www.cbs.dtu.dk/services/NetNGlyc/>, and three N-myristoylation sites (AA 45–50, 56–61, 102–107), although nobody has to our knowledge tested if these predicted post-translational modifications indeed occur *in vivo*. These data, to conclude, thus clearly suggest that not only the AA composition but also the tertiary organization of this protein has remained relatively constant in evolution and that *A. alternata* TCTP possibly shares the structural elements known to build individual domains possessing defined biochemical functions such as microtubule or Ca^{2+} binding (Hinojosa-Moya et al., 2008), do, on the other hand, however, not rule out the probability of possible physiological differences.

3.2. Expression and purification of recombinant *A. alternata* TCTP

Following an induction with 0.5 mM IPTG that results in the expression of the T₇ RNA polymerase which again is necessary to mediate the synthesis of heterologous inserts subcloned into the BamHI and EcoRI restriction sites of pHIS parallel 2 under control of the respective promoter within the *E. coli* strain BL21(DE3), the cDNA sequence encoding *A. alternata* TCTP was in a first step expressed as a hexahistidine-tagged fusion protein and then purified via fast-flow Ni^{2+} chelate affinity chromatography under native, non-denaturing conditions by gravity flow using a gradient elution system, resulting in a yield of 22.1 mg pure protein per liter culture. This substantial quantity was mainly achieved due to the bacterial production of recombinant *A. alternata* TCTP at low temperatures which avoided its accumulation in form of insoluble inclusion bodies and thereby circumvented a complicated *in vitro* refolding procedure to recover its biological activity (Thapa et al., 2008). The resulting 6×HIS-tagged fusion molecule harbours the extra 28 vector-encoded amino acids MSYYHHHHHHNYNIPTTENLYFQGAMGS (HIS-tag underlined), pos-

sesses a theoretically calculated molecular weight of 22.3 kDa and a *pI* value of 4.92 (which is slightly more basic than considered for the rnf allergen), respectively. The hexahistidine-tagged *A. alternata* TCTP, which was primarily present within the fraction eluted with 100 mM imidazole, appeared as a prominent band on a Coomassie-stained SDS-PAGE gel, with a considerably larger apparent molecular weight than the calculated one as has been noticed before (Rid et al., 2008; Sanchez et al., 1997).

Subsequently, its affinity tag was cleaved off by TEV protease, an enhanced, extremely site-specific enzyme that recognizes the seven AA sequence ENLYFQG present on pHIS parallel 2 and cuts between glutamine and glycine, and samples subjected to a second affinity chromatographic passage in the course of which further impurities as well as the via genetic engineering approaches itself 6×HIS-tagged protease were removed. The purified rnf *A. alternata* TCTP protein, which N-terminally concisely only carries the five amino acids GAMGS that obligatorily derive from the multiple cloning site and are not part of the natural protein, was found to be essentially depleted from any *E. coli* contaminants as determined by denaturing SDS-PAGE electrophoresis, followed by a silver staining (Fig. 4B).

Far-UV circular dichroism CD spectrometry measurements (Fig. 4A), a convenient technique for studying the conformation of polypeptides in solution and for assessing the structural integrity of recombinant proteins, revealed a typical and almost superimposable curve shape with comparable peak amplitudes for rnf *A. alternata*, *C. herbarum* (which has in previous studies already been shown to be biologically active as a histamine-releasing factor by measuring its *in vitro* capacity to induce mediator discharge from dextran sedimented human basophils; Rid et al., 2008) as well as human TCTP (Fig. 4A), indicating that they do not exist as random coils, but are natively-like folded and indeed possess a close secondary structure similarity. Each final CD profile obtained after baseline subtraction monitored under identical conditions represents an average of five consecutive scans.

3.3. Immunoreactivity and cross-reactivity of recombinant *A. alternata* TCTP

The importance of *A. alternata* TCTP as a novel ascomycete allergen as well as a probable cross-reactivity with *C. herbarum* TCTP and its human TCTP homologue as has already been reported for several other mold proteins, was in a further step assessed by subjecting rnf-AaTCTP to immunoblot analysis in the course of which serum IgE obtained from a variety of selected mold allergic individuals was detected by autoradiography using an ^{125}I -labeled secondary rabbit anti-human IgE antibody. Additionally to the routinely performed negative controls where membrane strips were incubated with serum of one non-atopic individual (NHS) or the secondary antibody only, an extra reaction with serum of a birch pollen allergic patient with an extremely high IgE titer was performed (data not shown), thereby excluding any artificial false-positive background reactivity due to unspecific binding of IgE antibodies. The ability of our newly identified *A. alternata* TCTP protein that was purified by standard affinity chromatography approaches as denoted above to bind IgE antibodies in our *in vitro* immunoblots provides significant evidence for its allergenicity, albeit its prevalence of IgE reactivity is merely approximately 4%, thus representing a minor fungal allergen. Interestingly, all these nine individuals presented in Fig. 5 also react with *C. herbarum* TCTP and five of them are even responding to human TCTP, a result that could be comparably reproduced with the affinity-tagged fusion proteins (data not shown), implying that the 6 $\times$ HIS affinity tag neither introduces new immunoreactive epitopes nor influences the IgE antibody binding by disrupting the existing antigenic determinants via conformational changes.

By performing so-called inhibition immunoblot assays, we in a next step intended to investigate whether *A. alternata* TCTP, *C. herbarum* TCTP and human TCTP actually share common, IgE binding cross-reactive epitopes. Whereas for example the serum of patient 1 upon preabsorption with 100 μg rnf human TCTP was not obviously able to reduce the binding of its IgE antibodies to *A. alternata* or *C. herbarum* TCTP, the same serum preincubated with the equal amount of either rnf *A. alternata* or *C. herbarum* TCTP could, conversely, completely inhibit antibody binding to the human protein as evident from Fig. 6. Besides employing both positive and negative control reactions (including the same serum without the respective pretreatment as well as sera of a birch pollen allergic patient and of a non-allergic individual, data not shown in detail) that again provided the presumed results, (i) a complete disappearance of the particular protein bands could be observed when the purified rnf *A. alternata* TCTP transferred to the PVDF mem-

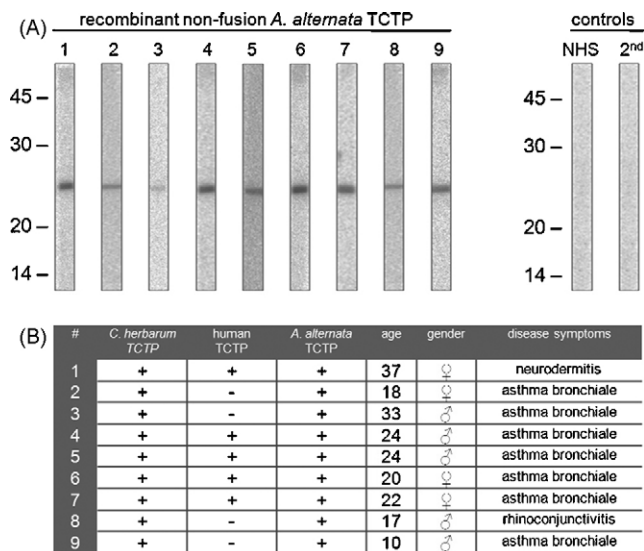


Fig. 5. Immunoblot analysis for specific IgE antibodies against rnf *A. alternata* TCTP. (A) A selection of nine patients with positive IgE reactivity towards *A. alternata* TCTP as detected by autoradiography. Negative control experiments included serum of a non-atopic individual (NHS), a birch pollen allergic patient and usage of 2nd antibody only (^{125}I -labeled-rabbit anti-human IgE antibody) and show no unspecific background reactivity. Molecular mass standards in kDa are shown on the left side. (B) List of the corresponding patients exhibiting positive IgE reactivity towards *A. alternata* TCTP, supplemented by a short summary of cross-reactivity with *C. herbarum* and eventually human TCTP, their age, gender and allergic symptoms (with special emphasis on asthma bronchiale).

brane was tested with serum preblocked with rnf-AaTCTP itself and *vice versa* (self-depletion), an important prerequisite for the reliable interpretation of these data, (ii) and *A. alternata* TCTP depleted serum could, in contrast to serum preincubated with *C. herbarum* TCTP that yet exhibited a faint residual IgE reactivity when analyzing blotted *A. alternata* TCTP, entirely inhibit IgE binding to *C. herbarum* TCTP. Comparable results were obtained with the sera of two further individuals (patients 4 and 6, data not shown). We can hence deduce from these immunoblot experiments that *A. alternata* TCTP indeed represents a novel, cross-reactive fungal “pan-allergen”, and although both *C. herbarum* and *A. alternata* TCTP actually share the majority of their IgE epitopes, the *A. alternata* allergen apparently possesses more antigenic determinants than its *C. herbarum* homologue and the respective *C. herbarum* protein still additional ones with respect to human TCTP. Findings rather similar to the above presented data, in particular that *C. herbarum* TCTP comprises more antigenic determinants than its human homologue as seems logical and realistic in regards of the fact that mold allergic individuals in the course of their deteriorating disease symptoms seldomly exhibit co-sensitization but instead rather cross-reactivity with the respective human counterparts, were previously already obtained through enzyme-linked immunosorbent assays and especially inhibition-ELISA experiments by our group (Rid et al., 2008).

Advanced bioinformatical and statistical investigations that were performed using BepiPred 1.0 as well as the BCPREDS Server 1.0 which in short examine actual secondary structure elements including hydrophilic, flexible or surface-protected domains within a given sequence and the frequency of certain amino acid residues therein that on the other hand have previously been analyzed to appear in a variety of known antigenic determinant regions (Kurup and Banerjee, 2000; Larsen et al., 2006), have in this context located two main putative linear continuous B-cell epitopes on *A. alternata* TCTP, namely ITVGESFDGTGANASAEQEEGAEDSAET and GLKKKGADATIKDFETKASGY. As cursorily depicted in Fig. 3B, these 2 mapped epitopes consist of (i) AA that are to a large extent

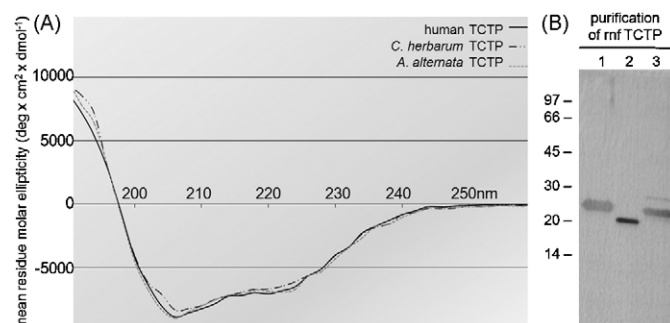


Fig. 4. Characterization of recombinant *A. alternata* TCTP. (A) Circular dichroism CD spectra of affinity-purified rnf *A. alternata*, *C. herbarum* and human TCTP that were recorded at wavelengths between 190 and 260 nm in 10 mM sodium phosphate buffer. Each spectrum represents an average of five consecutive scans. Final curves were obtained after subtraction of the baseline that was monitored under identical experimental conditions. (B) Silver-stained 13.5% SDS-PAGE gel of 5 μg purified, TEV-digested, rnf *C. herbarum* TCTP (1), human TCTP (2) and *A. alternata* TCTP (3). Molecular mass standards (M) in kDa are indicated on the left side.

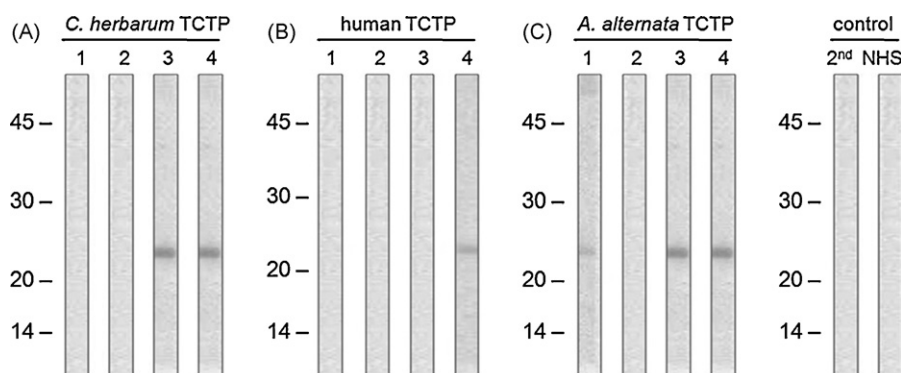


Fig. 6. Inhibition immunoblots establish a significant cross-reactivity between *A. alternata*, *C. herbarum* TCTP and human TCTP. (A) rnf *C. herbarum* TCTP blotted on a PVDF membrane was probed with (1) serum of patient 1 preincubated with 100 μ g *Cl*aTCTP (self-depletion), (2) 100 μ g *Aa*TCTP, (3) human TCTP and (4) BSA as a positive control. (B) Human TCTP transferred to a PVDF membrane was analyzed using the same serum preincubated with (1) *Cl*aTCTP, (2) *Aa*TCTP, (3) human TCTP (self-depletion) and (4) BSA. (C) Accordingly, *A. alternata* TCTP that was separated via SDS-PAGE and blotted on a PVDF membrane was tested with serum preabsorbed with (1) *Cl*aTCTP, (2) 100 μ g *Aa*TCTP (self-depletion), (3) 100 μ g human TCTP and (4) BSA. Negative control experiments included serum of a non-atopic individual (NHS) and usage of 2nd antibody only. Molecular mass standards in kDa are shown on the left side. These data show that patient 1 is primarily sensitized against fungal TCTP.

solvent-exposed and thus accessible for antigen-antibody interactions within the native protein, and (ii) reveal 68% identity and 84% similarity to *C. herbarum* TCTP as an essential necessity for their actual cross-reactivity. However, their direct involvement in IgE antibody binding, Fc ϵ RI crosslinking or mediator discharge by means of immunoblotting, inhibition experiments, ELISA testings or *in vitro* histamine-release studies in comparison to the respective full-length molecules remains to be demonstrated in future studies.

4. Discussion

Although several longitudinal epidemiologic population surveys have investigated the prevalence of sensitization to fungal, especially *A. alternata* and *C. herbarum*, allergens in different countries so far, its exact frequency is difficult to be reliably established because the inconsistency of commercial extracts used within routine assessments (debated below) often generates false-negative outcomes that most probably underestimate the precise values (Breitenbach and Simon-Nobbe, 2002; Bush et al., 2006; Crameri et al., 2006; Horner et al., 1995; Kurup et al., 2000; Simon-Nobbe et al., 2008). Large-scale analyses from various locations worldwide including, e.g., several inner cities of the United States, southern Australia, New Zealand or France as part of the European Community Respiratory Health Evaluation (Black et al., 2000; Downs et al., 2001; Eggleston, 2007; Gergen et al., 1987; Neukirch et al., 1999; Salo et al., 2006; Zureik et al., 2002) have, nevertheless, indicated (i) that sensitization against *Alternaria* species is closely linked to the development, persistence as well as severity of asthma, (ii) that within susceptible individuals, exposure to atmospheric *Alternaria* spores can be reliably correlated with enhanced airway responsiveness to histamine, bronchodilator usage and finally increased emergency department visits and (iii) that more than 54% of patients admitted to an intensive care unit during acute asthma attacks appear to have a positive result upon skin prick testing for 1 or more mold allergens (including *A. alternata*, *C. cladosporioides*, and *E. nigrum*) compared to only 30% in individuals suffering from simply milder forms of disease, whereas no statistically significant interrelation between asthma bronchiale and sensitization to cat dander, pollen, grass or house dust mite allergens was recorded (Bush and Prochnau, 2004; Delfino et al., 1996; Karihaloo et al., 2002). Numerous *A. alternata* allergens have been purified and characterized either by conventional fractionation methodologies or by employing newer molecular biology techniques since the 1990s, including Alt a 1 (28 kDa; De Vouge et al., 1998), the secreted

species-specific major allergen protein for which a number of variants as well as isoforms have been reported, protein disulfide isomerase (Alt a 4, 57 kDa; Achatz et al., 1995), YCP4 homologue (flavodoxin, Alt a 7, 22 kDa; Achatz et al., 1995), NADP-dependent mannitol dehydrogenase (Alt a 8, 29 kDa; Schneider et al., 2006), aldehyde dehydrogenase (Alt a 10, 53 kDa; Achatz et al., 1995), enolase (Alt a 6, 45 kDa; Simon-Nobbe et al., 2000), glutathione S-transferase (Alt a 13, 26 kDa; Shankar et al., 2006), heat shock protein 70 (Alt a 3, 70 kDa; De Vouge et al., 1998), acidic ribosomal phosphoproteins P₁ and P₂ (Alt a 12 and Alt a 5, 11 kDa; Achatz et al., 1995), nuclear transport factor 2 (Alt a NTF2, 13.7 kDa; Weichel et al., 2003) and, as cursorily treated in the current study, TCTP. The majority of them are intracellular housekeeping proteins characterized by the presence of cross-reactive epitopes which they share with the homologous *C. herbarum* allergens and to a certain extent also with the corresponding *A. fumigatus*, *Candida albicans*, *Penicillium citrinum*, *Malassezia sympodialis* as well as *S. cerevisiae* proteins (Simon-Nobbe et al., 2008).

TCTP itself, also designated as fortilin, HRF (histamine-releasing factor), Tma19 (translation-machinery associated) or *S. cerevisiae* MMI1 (microtubule and mitochondria interacting protein), was initially discovered as a molecule especially abundant in tumor cell lines as well as markedly overexpressed in tumor biopsy specimens and has been investigated for practically nearly 20 years now, yielding a gradually more coherent picture of its for a long time incompletely understood principal biochemical activities (Bommer and Thiele, 2004). TCTP is ubiquitously expressed in all eukaryotic organisms with levels that vary depending on the cell/tissue type, the developmental state and on a variety of extracellular signals and changing physiological parameters as has been demonstrated in several experimental settings (Bommer and Thiele, 2004; Bonnet et al., 2000; Hsu et al., 2007; Mak et al., 2007; Oikawa et al., 2002; Tuynder et al., 2004). Immunofluorescence microscopy has shown that TCTP binds to microtubules during G₁, S, G₂ and early M phase of the eukaryotic cell cycle and is detached from the spindle upon the meta- to anaphase transition to promote an increase in cytoskeletal dynamics (Gachet et al., 1999; Yarm, 2002). A TCTP overexpression in mammalian cells therefore leads to a slow growth phenotype, consequently to alterations in the cell morphology and to a delay in cell-cycle progression/cell division. Additional investigations implicate an involvement of TCTP in cytosolic ribosome actions and especially the translational elongation reaction via interaction with both eEF1A and its guanine nucleotide exchange factor eEF-1 δ (Cans et al., 2003; Langdon et al., 2004). Strikingly, TCTP is also involved in the prevention

of cell death by (i) modulating the anti-apoptotic activity Mcl-1 via interfering with its ubiquitin-mediated proteasomal degradation, probably by masking its ubiquitination sites upon formation of a functional complex (Liu et al., 2005), (ii) through the interaction with Bcl-xL (Yang et al., 2005), (iii) by inserting into the mitochondrial membrane and in this manner hindering Bax from undergoing dimerization that is needed for the formation of cytotoxic pores (Susini et al., 2008), (iv) by scavenging elevated Ca^{2+} levels within the cytosol (Graidist et al., 2007) and (v) by destabilizing TSC-22 (transforming growth factor β stimulated clone-22) (Lee et al., 2008), a pro-apoptotic factor identified in many cancer cells. On the other hand, TCTP moreover displays cytokine-like activities because it behaves as a B-cell growth factor stimulating enhanced MHCII expression and immunoglobulin production (Kang et al., 2001), induces the secretion of IL-4, IL-13 and histamine from basophilic granulocytes (MacDonald, 1997; MacDonald et al., 1995; Schroeder et al., 1997), acts as a chemoattractant for eosinophils and promotes their IL-8 and GM-CSF release (Bheekha-Escura et al., 2000), implying that it induces a complex array of responses at the sites of allergic inflammation and rather likely participates in the late phase of allergic inflammatory processes or in the development of chronic allergic disease. Evidently, in higher organisms which have the ability to mount a specific immune response, this multifaceted protein has acquired a second function in the adaptive immune system. Despite its original designation as an IgE-dependent histamine-releasing factor by MacDonald et al. (1995), supplementary work by the same research group revealed that TCTP is actually not dependent on the IgE molecule for its biological functions but rather exerts its effects away from the classical signal transduction cascade by binding to a distinct and to date still speculative cell surface structure (Bheekha-Escura et al., 1999; MacDonald and Vonakis, 2002). Because TCTP lacks any known N-terminal hydrophobic leader sequence characteristic for secreted proteins and is therefore unlikely to follow the typical secretion pathway, it is not proven how the protein is actually exported and finally occurs in the serum to exert its extracellular activity on neighbouring cells (Bommer and Thiele, 2004), however, Amzallag et al. (2004) have as a possibly first hint reported that TCTP as well as TSAP6, a 5–6 transmembrane protein, interact in the yeast two-hybrid system and co-localize in small secreted vesicles called exosomes. TCTP has furthermore also been characterized from various parasitic worms like *P. falciparum*, *S. mansoni*, *W. bancrofti* and *B. malayi* where it plays a role in parasite survival and thereby initiation of pathology because entry of the infective stages from the insect vector to a warm-blooded organism triggers the enhanced expression of the previously translationally repressed TCTP that is finally secreted into the host where it is able to stimulate histamine release from basophils as well as to cause inflammatory infiltration of eosinophils and their IL-8 secretion (Bhisutthibhan et al., 1998; Gnanasekar et al., 2002; MacDonald et al., 2001; Rao et al., 2002). It has been speculated that the vasodilatory effects of histamine and possibly other vasoactive amines released during the infection might facilitate the parasites to circulate through narrow blood vessels (Fujita et al., 2008; MacDonald et al., 2001).

Looking in more detail at the clinical history of our nine mold allergic individuals (Fig. 5B) who have within this respective study been tested to positively recognize both the rnf *A. alternata* and *C. herbarum* TCTP proteins in standard Western Blots and inhibition approaches reveals a strong association with relatively high total IgE levels and, even more importantly, besides suffering from typical symptoms as rhinitis, conjunctivitis or occasionally atopic dermatitis, with severe bronchial asthma in seven out of nine cases (77.8%). Interestingly, five patients (Fig. 5B) exhibiting a quite strong IgE reactivity to *A. alternata* and *C. herbarum* TCTP also display a significant cross-reactivity to the human homologue, and the supplementary observation that serum preincubation with AaTCTP

or ClaTCTP could completely inhibit binding to human TCTP, but not *vice versa* (Fig. 6), suggests that these individuals were most likely primarily immunized against the fungal molecules and not in parallel, independently co-sensitized against the human protein (Rid et al., 2008). Bioinformatical investigations and *ab initio* three-dimensional models (data not shown in detail) in this context predict that *A. alternata*, *C. herbarum* and human TCTP display a rather similar, evolutionarily highly conserved three domain architecture and as a result possess analogous B-cell epitopes which probably lead to the observed immunological cross-reactivity (Bommer and Thiele, 2004; Thaw et al., 2001). Our perceived association of IgE cross-reactivity between ascomycete allergens and their human homologues (which do not as perhaps anticipated induce immunologic tolerance) could analogously, both *in vitro* and *in vivo* (skin prick tests, SPT), already be repeatedly demonstrated for *A. fumigatus* stress-inducible manganese-dependent superoxide dismutase (MnSOD), acidic ribosomal phosphoprotein 2, thioredoxin and cyclophilins which are all intracellular molecules showing sequence and structural identities of more than 50–70% to the corresponding human proteins (molecular mimicry), suggesting that cross-/auto-reactivity and sequence similarity seem to be well correlated (Appenzeller et al., 1999; Cramer et al., 1996; Fluckiger et al., 2002; Mayer et al., 1999). Schmid-Grendelmeier et al. (2005) in addition demonstrated that (i) 36% of patients suffering from skin colonization of *M. sympodialis* were SPT positive to *M. sympodialis* extract, to purified recombinant human MnSOD and to structurally related MnSODs, (ii) the severe form of follow-on atopic eczema was accompanied by chronic inflammation and cell lysis, (iii) human MnSOD was in histochemical specimens locally overexpressed in lesional areas as a reaction to oxidative stress or due to mechanical trauma, and (iv) a liberation of human MnSOD in this scenario led to binding of the *M. sympodialis* MnSOD-specific antibodies, aggravation of disease and in the long run to symptoms which were independent of the presence of the environmental allergen (Cramer et al., 2006; Rottem and Shoenfeld, 2003). Similarly, one could imagine that cytoplasmic molecules that are under normal physiologic conditions unlikely to be accessible for antigen-antibody interactions may be continuously released by lysed lung cells in the progression of severe long-lasting inflammatory processes, resulting in the maintenance and finally the clinical exacerbation of the allergic reaction even in the absence of external antigen challenge (Aalberse et al., 2001; Cramer et al., 1996; Cramer et al., 2006; Rid et al., 2008).

Though allergic symptoms can be relieved through antihistaminic medications, the only causative treatment is up to now specific immunotherapy (SIT), a controlled administration of gradually increasing doses of the disease-eliciting allergen to induce a state of unresponsiveness to subsequent antigen exposure. SIT is, as far as fungal allergy is concerned, however, still hampered by the inconsistency of the routinely used industrially available crude mold extracts due to problems in manufacturing as well as standardizing these solutions, resulting in a variable allergen composition (contaminated with a variety of unwanted non-allergenic components) upon the comparison of extracts of different suppliers or distinct batches of the same company what again limits their broad application (Cramer et al., 2006; Esch, 2004; Horner et al., 1995; Kurup et al., 2000; Vailes et al., 2001). Despite the fact that immunotherapy with crude fungal extracts is for these reasons problematic, successful and clinically effective hyposensitization trials with *A. alternata*, *A. fumigatus* as well as *C. herbarum* extracts have been reviewed (Bonifazi, 1994; Lizaso et al., 2008; Mallin, 1992; Tabar et al., 2008), but are in most countries not recommended because (i) an individual might *de novo* develop IgE antibodies against further components present in the crude extract, thus paradoxically broadening his sensitization spectrum, (ii) this treatment cannot be applied according to the patient's individual

reactivity profile, and (iii) the required effective therapeutic doses can often not be reached since severe therapy-induced side effects may occur (Simon-Nobbe et al., 2008). These problems could, nevertheless, be overcome by the (i) use of ultrapure recombinant allergens that can be produced in unlimited amounts in both pro- and eukaryotic expression systems and reproducibly standardized with the help of mass spectrometry, determination of T-cell reactivity, histamine-release assays or circular dichroism, as well as by (ii) the administration of genetically engineered molecules which aim at maintaining the immunogenicity of an antigen while reducing or even avoiding its capacity to bind allergen-specific IgE, thereby opening new vaccination possibilities (Ferreira et al., 2004; Niederberger and Valenta, 2004; Valenta, 2002; Valenta and Niederberger, 2007). A first clinical study by Unger et al. (1999) utilizing Alt a 1 and enolase cloned into the non-fusion vector pMW172 and purified by inclusion body preparation, ammonium sulfate precipitation and DEAE-chromatography has in this context already clearly shown that recombinant allergens achieve a higher specificity as well as sensitivity than commercial mold extracts (which in part failed to correctly identify the fungal allergy) and that a combination of these two molecules, perhaps supplemented with a few further allergens, represents a promising approach for diagnosis and therapy of *A. alternata* allergy. As trends go noticeably in the direction of component-resolved, patient-tailored approaches (Cromwell et al., 2004; Ferreira et al., 2004), recombinant *A. alternata* TCTP has the potential to contribute to an improved diagnosis of immediate hypersensitivity reactions in mold allergic patients and to offer new, beneficial immunotherapeutic strategies to better control fungal allergic disease.

Acknowledgements

We are grateful to the Austrian Science Fund FWF (Vienna, Austria) for grant S9302-B05 (to M.B.), to the EC (Brussels, Europe) for project MIMAGE (contract no. 512020; to M.B.), to the Austrian Academy of Sciences (Vienna, Austria) for a DOC-fORTE stipend (to R.R.) as well as to Peter Sheffield for providing us the fusion vector pHIS parallel 2.

References

- Aalberse, R.C., Akkerdaas, J., van Ree, R., 2001. Cross-reactivity of IgE antibodies to allergens. *Allergy* 56, 478–490.
- Achatz, G., Oberkofler, H., Lechenauer, E., Simon, B., Unger, A., Kandler, D., Ebner, C., Prillinger, H., Kraft, D., Breitenbach, M., 1995. Molecular cloning of major and minor allergens of *Alternaria alternata* and *Cladosporium herbarum*. *Mol. Immunol.* 32, 213–227.
- Achatz, G., Oberkofler, H., Lechenauer, E., Simon, B., Unger, A., Kandler, D., Ebner, C., Prillinger, H., Kraft, D., Breitenbach, M., 1996. Molecular characterization of *Alternaria alternata* and *Cladosporium herbarum* allergens. *Adv. Exp. Med. Biol.* 409, 157–161.
- Al-Samarrai, T.H., Schmid, J., 2000. A simple method for extraction of fungal genomic DNA. *Lett. Appl. Microbiol.* 30, 53–56.
- Amzallag, N., Passer, B.J., Allanic, D., Segura, E., Thery, C., Goud, B., Amson, R., Telerman, A., 2004. TSAP6 facilitates the secretion of translationally controlled tumor protein/histamine-releasing factor via a nonclassical pathway. *J. Biol. Chem.* 279, 46104–46112.
- Appenzeller, U., Meyer, C., Menz, G., Blaser, K., Cramer, R., 1999. IgE-mediated reactions to autoantigens in allergic diseases. *Int. Arch. Allergy Immunol.* 118, 193–196.
- Bairoch, A., Apweiler, R., Wu, C.H., Barker, W.C., Boeckmann, B., Ferro, S., Gasteiger, E., Huang, H., Lopez, R., Magrane, M., Martin, M.J., Natale, D.A., O'Donovan, C., Redaschi, N., Yeh, L.S., 2005. The Universal Protein Resource (UniProt). *Nucleic Acids Res.* 33, D154–D159.
- Baxter, N.J., Thaw, P., Higgins, L.D., Sedelnikova, S.E., Bramley, A.L., Price, C., Waltho, J.P., Craven, C.J., 2000. Backbone NMR assignment of the 19 kDa translationally controlled tumor-associated protein p23fyp from *Schizosaccharomyces pombe*. *J. Biomol. NMR* 16, 83–84.
- Bheekha-Escura, R., Chance, S.R., Langdon, J.M., MacGlashan Jr., D.W., MacDonald, S.M., 1999. Pharmacologic regulation of histamine release by the human recombinant histamine-releasing factor. *J. Allergy Clin. Immunol.* 103, 937–943.
- Bheekha-Escura, R., MacGlashan, D.W., Langdon, J.M., MacDonald, S.M., 2000. Human recombinant histamine-releasing factor activates human eosinophils and the eosinophilic cell line AML14-3D10. *Blood* 96, 2191–2198.
- Bhisutthibhan, J., Pan, X.Q., Hossler, P.A., Walker, D.J., Yowell, C.A., Carlton, J., Dame, J.B., Meshnick, S.R., 1998. The *Plasmodium falciparum* translationally controlled tumor protein homolog and its reaction with the antimalarial drug artemisinin. *J. Biol. Chem.* 273, 16192–16198.
- Black, P.N., Udy, A.A., Brodie, S.M., 2000. Sensitivity to fungal allergens is a risk factor for life-threatening asthma. *Allergy* 55, 501–504.
- Bommer, U.A., Thiele, B.J., 2004. The translationally controlled tumour protein (TCTP). *Int. J. Biochem. Cell Biol.* 36, 379–385.
- Bonifazi, F., 1994. Immunotherapy in pollen and mould asthma. *Monaldi Arch. Chest Dis.* 49, 150–153.
- Bonnet, C., Perret, E., Dumont, X., Picard, A., Caput, D., Lenaers, G., 2000. Identification and transcription control of fission yeast genes repressed by an ammonium starvation growth arrest. *Yeast* 16, 23–33.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Breitenbach, M., Simon-Nobbe, B., 2002. The allergens of *Cladosporium herbarum* and *Alternaria alternata*. *Chem. Immunol.* 81, 48–72.
- Burge, H.A., 2002. An update on pollen and fungal spore aerobiology. *J. Allergy Clin. Immunol.* 110, 544–552.
- Bush, R.K., Portnoy, J.M., Saxon, A., Terr, A.I., Wood, R.A., 2006. The medical effects of mold exposure. *J. Allergy Clin. Immunol.* 117, 326–333.
- Bush, R.K., Prochnau, J.J., 2004. *Alternaria*-induced asthma. *J. Allergy Clin. Immunol.* 113, 227–234.
- Cans, C., Passer, B.J., Shalak, V., Nancy-Portebois, V., Crible, V., Amzallag, N., Allanic, D., Tufino, R., Argenti, M., Moras, D., Fiucci, G., Goud, B., Mirande, M., Amson, R., Telerman, A., 2003. Translationally controlled tumor protein acts as a guanine nucleotide dissociation inhibitor on the translation elongation factor eEF1A. *Proc. Natl. Acad. Sci. U.S.A.* 100, 13892–13897.
- Cramer, R., Faith, A., Hemmann, S., Jaussi, R., Ismail, C., Menz, G., Blaser, K., 1996. Humoral and cell-mediated autoimmunity in allergy to *Aspergillus fumigatus*. *J. Exp. Med.* 184, 265–270.
- Cramer, R., Weichel, M., Fluckiger, S., Glaser, A.G., Rhyner, C., 2006. Fungal allergies: a yet unsolved problem. *Chem. Immunol. Allergy* 91, 121–133.
- Cromwell, O., Suck, R., Kahlert, H., Nandy, A., Weber, B., Fiebig, H., 2004. Transition of recombinant allergens from bench to clinical application. *Methods* 32, 300–312.
- De Vouge, M.W., Thaker, A.J., Zhang, L., Muradia, G., Rode, H., Vijay, H.M., 1998. Molecular cloning of IgE-binding fragments of *Alternaria alternata* allergens. *Int. Arch. Allergy Immunol.* 116, 261–268.
- Delfino, R.J., Coate, B.D., Zeiger, R.S., Seltzer, J.M., Street, D.H., Koutrakis, P., 1996. Daily asthma severity in relation to personal ozone exposure and outdoor fungal spores. *Am. J. Respir. Crit. Care Med.* 154, 633–641.
- Downs, S.H., Mitakakis, T.Z., Marks, G.B., Car, N.G., Belousova, E.G., Leuppi, J.D., Xuan, W., Downie, S.R., Tobias, A., Peat, J.K., 2001. Clinical importance of *Alternaria* exposure in children. *Am. J. Respir. Crit. Care Med.* 164, 455–459.
- Eggleston, P.A., 2007. The environment and asthma in US inner cities. *Chest* 132, 782S–788S.
- Esch, R.E., 2004. Manufacturing and standardizing fungal allergen products. *J. Allergy Clin. Immunol.* 113, 210–215.
- Feng, Y., Liu, D., Yao, H., Wang, J., 2007. Solution structure and mapping of a very weak calcium-binding site of human translationally controlled tumor protein by NMR. *Arch. Biochem. Biophys.* 467, 48–57.
- Ferreira, F., Wallner, M., Thalhammer, J., 2004. Customized antigens for desensitizing allergic patients. *Adv. Immunol.* 84, 79–129.
- Fluckiger, S., Scapozza, L., Mayer, C., Blaser, K., Folkers, G., Cramer, R., 2002. Immunological and structural analysis of IgE-mediated cross-reactivity between manganese superoxide dismutases. *Int. Arch. Allergy Immunol.* 128, 292–303.
- Frew, A.J., 2004. Mold allergy: some progress made, more needed. *J. Allergy Clin. Immunol.* 113, 216–218.
- Fujita, T., Felix, K., Pinkaew, D., Hutadilok-Towatana, N., Liu, Z., Fujise, K., 2008. Human fortilin is a molecular target of dihydroartemisinin. *FEBS Lett.* 582, 1055–1060.
- Gachet, Y., Tournier, S., Lee, M., Lazaris-Karatzas, A., Poulton, T., Bommer, U.A., 1999. The growth-related, translationally controlled protein P23 has properties of a tubulin binding protein and associates transiently with microtubules during the cell cycle. *J. Cell Sci.* 112 (Pt 8), 1257–1271.
- Gasteiger, E., Jung, E., Bairoch, A., 2001. SWISS-PROT: connecting biomolecular knowledge via a protein database. *Curr. Issues Mol. Biol.* 3, 47–55.
- Gergen, P.J., Turkeltaub, P.C., Kovar, M.G., 1987. The prevalence of allergic skin test reactivity to eight common aeroallergens in the U.S. population: results from the second National Health and Nutrition Examination Survey. *J. Allergy Clin. Immunol.* 80, 669–679.
- Gnanasekar, M., Rao, K.V., Chen, L., Narayanan, R.B., Geetha, M., Scott, A.L., Ramaswamy, K., Kaliraj, P., 2002. Molecular characterization of a calcium binding translationally controlled tumor protein homolog from the filarial parasites *Brugia malayi* and *Wuchereria bancrofti*. *Mol. Biochem. Parasitol.* 121, 107–118.
- Graidist, P., Yazawa, M., Tonganunt, M., Nakatomi, A., Lin, C.C., Chang, J.Y., Phongdara, A., Fujise, K., 2007. Fortilin binds Ca²⁺ and blocks Ca²⁺-dependent apoptosis in vivo. *Biochem. J.* 408, 181–191.
- Hinojosa-Moya, J., Xocostle-Cazares, B., Piedra-Ibarra, E., Mendez-Tenorio, A., Lucas, W.J., Ruiz-Medrano, R., 2008. Phylogenetic and structural analysis of translationally controlled tumor proteins. *J. Mol. Evol.* 66, 472–483.
- Horner, W.E., Helbling, A., Salvaggio, J.E., Lehrer, S.B., 1995. Fungal allergens. *Clin. Microbiol. Rev.* 8, 161–179.

- Hsu, Y.C., Chern, J.J., Cai, Y., Liu, M., Choi, K.W., 2007. *Drosophila* TCTP is essential for growth and proliferation through regulation of dRheb GTPase. *Nature* 445, 785–788.
- Jacob, B., Ritz, B., Gehring, U., Koch, A., Bischof, W., Wichmann, H.E., Heinrich, J., 2002. Indoor exposure to molds and allergic sensitization. *Environ. Health Perspect.* 110, 647–653.
- Kang, H.S., Lee, M.J., Song, H., Han, S.H., Kim, Y.M., Im, J.Y., Choi, I., 2001. Molecular identification of IgE-dependent histamine-releasing factor as a B cell growth factor. *J. Immunol.* 166, 6545–6554.
- Kapust, R.B., Tozser, J., Fox, J.D., Anderson, D.E., Cherry, S., Copeland, T.D., Waugh, D.S., 2001. Tobacco etch virus protease: mechanism of autolysis and rational design of stable mutants with wild-type catalytic proficiency. *Protein Eng.* 14, 993–1000.
- Karihaloo, C., Tovey, E.R., Mitakakis, T.Z., Duffy, D.L., Britton, W.J., 2002. Evidence for the genetic control of immunoglobulin E reactivity to the allergens of *Alternaria alternata*. *Clin. Exp. Allergy* 32, 1316–1322.
- Kurup, V.P., Banerjee, B., 2000. Fungal allergens and peptide epitopes. *Peptides* 21, 589–599.
- Kurup, V.P., Shen, H.D., Banerjee, B., 2000. Respiratory fungal allergy. *Microbes Infect.* 2, 1101–1110.
- Kurup, V.P., Shen, H.D., Vijay, H., 2002. Immunobiology of fungal allergens. *Int. Arch. Allergy Immunol.* 129, 181–188.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Langdon, J.M., Vonakis, B.M., MacDonald, S.M., 2004. Identification of the interaction between the human recombinant histamine releasing factor/translationally controlled tumor protein and elongation factor-1 delta (also known as eElongation factor-1B beta). *Biochim. Biophys. Acta* 1688, 232–236.
- Larsen, J.E., Lund, O., Nielsen, M., 2006. Improved method for predicting linear B-cell epitopes. *Immunome Res.* 2, 2.
- Lee, J.H., Rho, S.B., Park, S.Y., Chun, T., 2008. Interaction between fortilin and transforming growth factor-beta stimulated clone-22 (TSC-22) prevents apoptosis via the destabilization of TSC-22. *FEBS Lett.* 582, 1210–1218.
- Liu, H., Peng, H.W., Cheng, Y.S., Yuan, H.S., Yang-Yen, H.F., 2005. Stabilization and enhancement of the antiapoptotic activity of mcl-1 by TCTP. *Mol. Cell. Biol.* 25, 3117–3126.
- Lizaso, M.T., Tabar, A.I., Garcia, B.E., Gomez, B., Algorta, J., Asturias, J.A., Martinez, A., 2008. Double-blind, placebo-controlled *Alternaria alternata* immunotherapy: in vivo and in vitro parameters. *Pediatr. Allergy Immunol.* 19, 76–81.
- MacDonald, S.M., 1997. Human recombinant histamine-releasing factor. *Int. Arch. Allergy Immunol.* 113, 187–189.
- MacDonald, S.M., Bhisutthibhan, J., Shapiro, T.A., Rogerson, S.J., Taylor, T.E., Tembo, M., Langdon, J.M., Meshnick, S.R., 2001. Immune mimicry in malaria: plasmodium falciparum secretes a functional histamine-releasing factor homolog in vitro and in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10829–10832.
- MacDonald, S.M., Rafnar, T., Langdon, J., Lichtenstein, L.M., 1995. Molecular identification of an IgE-dependent histamine-releasing factor. *Science* 269, 688–690.
- MacDonald, S.M., Vonakis, B.M., 2002. Association of the Src homology 2 domain-containing inositol 5' phosphatase (SHIP) to releasability in human basophils. *Mol. Immunol.* 38, 1323–1327.
- Mak, C.H., Poon, M.W., Lun, H.M., Kwok, P.Y., Ko, R.C., 2007. Heat-inducible translationally controlled tumor protein of *Trichinella pseudospiralis*: cloning and regulation of gene expression. *Parasitol. Res.* 100, 1105–1111.
- Malling, H.J., 1992. Immunotherapy for mold allergy. *Clin. Rev. Allergy* 10, 237–251.
- Mayer, C., Appenzeller, U., Seelbach, H., Achatz, G., Oberkofler, H., Breitenbach, M., Blaser, K., Cramer, R., 1999. Humoral and cell-mediated autoimmune reactions to human acidic ribosomal P2 protein in individuals sensitized to *Aspergillus fumigatus* P2 protein. *J. Exp. Med.* 189, 1507–1512.
- Neukirch, C., Henry, C., Leynaert, B., Liard, R., Bousquet, J., Neukirch, F., 1999. Is sensitization to *Alternaria alternata* a risk factor for severe asthma? A population-based study. *J. Allergy Clin. Immunol.* 103, 709–711.
- Niederberger, V., Valenta, R., 2004. Genetically modified allergens. *Immunol. Allergy Clin. N. Am.* 24, 727–738.
- Oikawa, K., Ohbayashi, T., Mimura, J., Fujii-Kuriyama, Y., Teshima, S., Rokutan, K., Mukai, K., Kuroda, M., 2002. Dioxin stimulates synthesis and secretion of IgE-dependent histamine-releasing factor. *Biochem. Biophys. Res. Commun.* 290, 984–987.
- Portnoy, J.M., Barnes, C.S., Kennedy, K., 2004. Sampling for indoor fungi. *J. Allergy Clin. Immunol.* 113, 199, 189–98; quiz.
- Rao, K.V., Chen, L., Gnanasekar, M., Ramaswamy, K., 2002. Cloning and characterization of a calcium-binding, histamine-releasing protein from *Schistosoma mansoni*. *J. Biol. Chem.* 277, 31207–31213.
- Rid, R., Simon-Nobbe, B., Langdon, J., Holler, C., Wally, V., Poll, V., Ebner, C., Hemmer, W., Hawranek, T., Lang, R., Richter, K., MacDonald, S., Rinnerthaler, M., Laun, P., Mari, A., Breitenbach, M., 2008. *Cladosporium herbarum* translationally controlled tumor protein (TCTP) is an IgE-binding antigen and is associated with disease severity. *Mol. Immunol.* 45, 406–418.
- Rottem, M., Shoenfeld, Y., 2003. Asthma as a paradigm for autoimmune disease. *Int. Arch. Allergy Immunol.* 132, 210–214.
- Salo, P.M., Arbes Jr., S.J., Sever, M., Jaramillo, R., Cohn, R.D., London, S.J., Zeldin, D.C., 2006. Exposure to *Alternaria alternata* in US homes is associated with asthma symptoms. *J. Allergy Clin. Immunol.* 118, 892–898.
- Sanchez, H., Bush, R.K., 2001. A review of *Alternaria alternata* sensitivity. *Rev. Iberoam. Micol.* 18, 56–59.
- Sanchez, J.C., Schaller, D., Ravier, F., Golaz, O., Jaccoud, S., Belet, M., Wilkins, M.R., James, R., Deshusses, J., Hochstrasser, D., 1997. Translationally controlled tumor protein: a protein identified in several nontumoral cells including erythrocytes. *Electrophoresis* 18, 150–155.
- Schmid-Grendelmeier, P., Fluckiger, S., Disch, R., Trautmann, A., Wuthrich, B., Blaser, K., Scheynius, A., Cramer, R., 2005. IgE-mediated and T cell-mediated autoimmunity against manganese superoxide dismutase in atopic dermatitis. *J. Allergy Clin. Immunol.* 115, 1068–1075.
- Schneider, P.B., Denk, U., Breitenbach, M., Richter, K., Schmid-Grendelmeier, P., Nobbe, S., Himly, M., Mari, A., Ebner, C., Simon-Nobbe, B., 2006. *Alternaria alternata* NADP-dependent mannitol dehydrogenase is an important fungal allergen. *Clin. Exp. Allergy* 36, 1513–1524.
- Schroeder, J.T., Lichtenstein, L.M., MacDonald, S.M., 1997. Recombinant histamine-releasing factor enhances IgE-dependent IL-4 and IL-13 secretion by human basophils. *J. Immunol.* 159, 447–452.
- Shankar, J., Singh, B.P., Gaur, S.N., Arora, N., 2006. Recombinant glutathione-S-transferase a major allergen from *Alternaria alternata* for clinical use in allergy patients. *Mol. Immunol.* 43, 1927–1932.
- Sheffield, P., Garrard, S., Derewenda, Z., 1999. Overcoming expression and purification problems of RhoGDI using a family of “parallel” expression vectors. *Protein Expres. Purif.* 15, 34–39.
- Simon-Nobbe, B., Denk, U., Poll, V., Rid, R., Breitenbach, M., 2008. The spectrum of fungal allergy. *Int. Arch. Allergy Immunol.* 145, 58–86.
- Simon-Nobbe, B., Probst, G., Kajava, A.V., Oberkofler, H., Susani, M., Cramer, R., Ferreira, F., Ebner, C., Breitenbach, M., 2000. IgE-binding epitopes of enolases, a class of highly conserved fungal allergens. *J. Allergy Clin. Immunol.* 106, 887–895.
- Susini, L., Besse, S., Duflaut, D., Lespagnol, A., Beekman, C., Fiucci, G., Atkinson, A.R., Busso, D., Poussin, P., Marine, J.C., Martinou, J.C., Cavarelli, J., Moras, D., Amson, R., Telerman, A., 2008. TCTP protects from apoptotic cell death by antagonizing bax function. *Cell Death Differ.* 15, 1211–1220.
- Tabar, A.I., Lizaso, M.T., Garcia, B.E., Gomez, B., Echechipia, S., Aldunate, M.T., Madariaga, B., Martinez, A., 2008. Double-blind, placebo-controlled study of *Alternaria alternata* immunotherapy: clinical efficacy and safety. *Pediatr. Allergy Immunol.* 19, 67–75.
- Terr, A.I., 2004. Are indoor molds causing a new disease? *J. Allergy Clin. Immunol.* 113, 221–226.
- Thapa, A., Shahnawaz, M., Karki, P., Raj Dahal, G., Golam Sharoar, M., Yub Shin, S., Sup Lee, J., Cho, B., Park, I.S., 2008. Purification of inclusion body-forming peptides and proteins in soluble form by fusion to *Escherichia coli* thermostable proteins. *Biotechniques* 44, 787–796.
- Thaw, P., Baxter, N.J., Hounslow, A.M., Price, C., Waltho, J.P., Craven, C.J., 2001. Structure of TCTP reveals unexpected relationship with guanine nucleotide-free chaperones. *Nat. Struct. Biol.* 8, 701–704.
- Tuynder, M., Fiucci, G., Prieur, S., Lespagnol, A., Geant, A., Beaucourt, S., Duflaut, D., Besse, S., Susini, L., Cavarelli, J., Moras, D., Amson, R., Telerman, A., 2004. Translationally controlled tumor protein is a target of tumor reversion. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15364–15369.
- Unger, A., Stoger, P., Simon-Nobbe, B., Susani, M., Cramer, R., Ebner, C., Hintner, H., Breitenbach, M., 1999. Clinical testing of recombinant allergens of the mold *Alternaria alternata*. *Int. Arch. Allergy Immunol.* 118, 220–221.
- Vailes, L., Sridhara, S., Cromwell, O., Weber, B., Breitenbach, M., Chapman, M., 2001. Quantitation of the major fungal allergens, Alt a 1 and Asp f 1, in commercial allergenic products. *J. Allergy Clin. Immunol.* 107, 641–646.
- Valenta, R., 2002. Recombinant allergen-based concepts for diagnosis and therapy of type I allergy. *Allergy* 57 (Suppl 71), 66–67.
- Valenta, R., Niederberger, V., 2007. Recombinant allergens for immunotherapy. *J. Allergy Clin. Immunol.* 119, 826–830.
- Venugopal, T., 2005. Evolution and expression of translationally controlled tumour protein (TCTP) of fish. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 142, 8–17.
- Weichel, M., Schmid-Grendelmeier, P., Fluckiger, S., Breitenbach, M., Blaser, K., Cramer, R., 2003. Nuclear transport factor 2 represents a novel cross-reactive fungal allergen. *Allergy* 58, 198–206.
- Yan, L., Fei, K., Bridge, D., Sarrajs Jr., M.P., 2000. A cnidarian homologue of translationally controlled tumor protein (P23/TCTP). *Dev. Genes Evol.* 210, 507–511.
- Yang, Y., Yang, F., Xiong, Z., Yan, Y., Wang, X., Nishino, M., Mirkovic, D., Nguyen, J., Wang, H., Yang, X.F., 2005. An N-terminal region of translationally controlled tumor protein is required for its antiapoptotic activity. *Oncogene* 24, 4778–4788.
- Yarm, F.R., 2002. Plk phosphorylation regulates the microtubule-stabilizing protein TCTP. *Mol. Cell. Biol.* 22, 6209–6221.
- Zureik, M., Neukirch, C., Leynaert, B., Liard, R., Bousquet, J., Neukirch, F., 2002. Sensitisation to airborne moulds and severity of asthma: cross sectional study from European Community respiratory health survey. *BMJ* 325, 411–414.