



Cladosporium herbarum translationally controlled tumor protein (TCTP) is an IgE-binding antigen and is associated with disease severity[☆]

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Abstract

Cladosporium herbarum represents one of the most important world-wide occurring allergenic fungal species. The prevalence of IgE reactivity to *C. herbarum* in patients suffering from allergy varies between 5 and 30% in the different climatic zones. Since mold allergy has often been associated with severe asthma, along with other allergic symptoms, it is important to define more comprehensively the allergen repertoire of this ascomycete. In this context we are reporting our successful approach to identify, clone, produce as a recombinant protein, purify and further characterize a new *C. herbarum* allergen which is a close homolog of the human translationally controlled tumor protein (TCTP, also called histamine releasing factor, HRF). The immunoreactivity of both pure recombinant molecules was investigated by means of immunoblot analyses, enzyme-linked immunosorbent assays as well as histamine release studies. To summarize, IgE antibodies from five out of nine individuals recognized both the human and the fungal protein in immunoblots. The latter was able to cause histamine release from human basophils with about half the efficiency compared to its human homolog HRF. Cross-inhibition assays showed that the patients' IgEs recognize common epitopes on both the human and *C. herbarum* proteins, but however, only pre-incubation with *C. herbarum* TCTP could completely inhibit reactivity with HRF. Furthermore, it appears that patients reactive to TCTP have a higher probability to suffer from asthma than other allergic patients.

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Keywords: TCTP; Histamine releasing factor; *Cladosporium herbarum*; Mold; Allergy; Autoreactivity

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); *C. herbarum*, *Cladosporium herbarum*; ClaTCTP, *Cladosporium herbarum* translationally controlled tumor protein; EDTA, ethylene diamine tetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; GST, glutathione-S-transferase; HRF, histamine releasing factor; hTCTP, human translationally controlled tumor protein; IPTG, isopropyl- β -D-thiogalactopyranoside; NHS, normal human serum; PBS, phosphate buffered saline; PIPES, piperazine-N-N'-bis [2-ethane sulphonic acid]; RAST, radioallergosorbent testing; rnf, recombinant non-fusion protein; TCTP, translationally controlled tumor protein; TEV, tobacco etch virus; T_M , melting temperature

[☆] Nucleotide and amino acid sequence information of ClaTCTP are available on the GenBank Sequence Database under accession number [AY349609](https://www.ncbi.nlm.nih.gov/nuclot/AY349609).

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1. Introduction

The small (19 kDa) and versatile protein TCTP has been highly conserved during the evolution of eukaryotes. It exhibits amino acid sequence identities of over 40% between distantly related species (Bommer and Thiele, 2004; Venugopal, 2005). To date, this protein family has been partly characterized with respect to biochemical activity, interaction partners, physiological function and subcellular or extracellular localization. Accordingly, several different names have been given to its orthologous members, among them “translationally controlled tumor protein” (TCTP), because the protein was originally found to be highly expressed in human tumors (Gachet et al., 1999; Tuynder et al., 2004; Yang et al., 2005). Other names include for instance “histamine releasing factor” (HRF), describing its extracellular function in histamine release as well as IL-4/IL-13 secretion from basophils (Langdon and MacDonald, 2006; MacDonald et al., 1995; Schroeder et al., 1996) and a distinct role as a cytokine stimulating B-cells (Kang et al., 2001). Both processes are possibly restricted to mammals and are highly relevant for human allergic disease. “Tma19p” on the other hand refers to an involvement in the elongation step of protein synthesis and to an interaction with both eEF1A (a small G protein and weak GTPase) and eEF1B β , its guanine nucleotide exchange factor. Since TCTP inhibits the latter, it is also called a “GTP/GDP dissociation inhibitor” and this function seems to be well conserved between yeast and human cells (Cans et al., 2003; Fleischer et al., 2006; Langdon et al., 2004). The yeast homolog of TCTP, “Mmi1p” (microtubule and mitochondria interacting), was shown to be glutathionylated in oxidative stress conditions (Shenton and Grant, 2003) and to be transferred from its cytoplasmic location to the mitochondria (Rinnerthaler et al., 2006). Moreover, the yeast Mmi1 deletion mutation was revealed to be hypersensitive to benomyl, which suggests an interaction of Mmi1p with microtubules (Rinnerthaler et al., 2006) as has been also demonstrated in human cells (Gachet et al., 1999). Finally, the term “fortilin” describes the antiapoptotic function of TCTP, which is exerted by interacting with and stabilizing Mcl1 as was first shown in tumor cells of myeloid leukaemia patients (Li et al., 2001; Liu et al., 2005; Zhang et al., 2002). Inhibiting TCTP synthesis by RNAi techniques greatly increases the frequency of tumor reversion, which is in accordance with the antiapoptotic action of the protein (Tuynder et al., 2004). Spontaneous revertants of tumor cell lines in many cases display a greatly reduced expression of the TCTP protein (Tuynder et al., 2002).

The structure of *S. pombe* TCTP was in recent years determined by NMR analysis (Thaw et al., 2001) and *ab initio* structure prediction of the *S. cerevisiae* protein revealed a very similar three-domain organization (Rinnerthaler et al., 2006). Structural features can in part be correlated with the functions just described. The protein consists of three clearly defined domains: an α -helical calcium binding domain that interacts with microtubules, a β -structured core domain that has been shown to interact with eEF1B β , and a flexible loop. The last two domains comprise the TCTP-signature. The single cysteine which is glutathionylated upon oxidative stress in yeast

(Rinnerthaler et al., 2006; Shenton and Grant, 2003), and the serine residue which is phosphorylated in the human system by a polo-like kinase (PLK) (Yarm, 2002) reside in the flexible loop. It seems that both postsynthetic modifications are not conserved in evolution, because glutathionylation and mitochondrial transfer were not found in human cells (M. Jendrach, pers. comm.) and the serine residue which is phosphorylated in the human system is not present in the yeast sequence. Both the transcript (Velculescu et al., 1997) and the protein (Fleischer et al., 2006) occur with very high abundance in rapidly growing, but not in starved or otherwise stressed cells, a very plausible finding considering the role of the protein in translation.

We are showing here that TCTP of the allergenic fungus *Cladosporium herbarum* is recognized by IgE antibodies of patients allergic to this ascomycete. We expressed and characterized the human and the *C. herbarum* protein in *E. coli*, and demonstrated that the fungal protein is active in *in vitro* histamine release assays. About 50% of the patients who recognized this minor fungal allergen also recognize human TCTP (HRF) in IgE immunoblots.

2. Materials and methods

2.1. Patients and sera

Sera were supplied by three Austrian allergy clinics, the Department of Dermatology, St. Johannis-Spital, Salzburg, the Allergieambulatorium Reumannplatz, Vienna, as well as the Floridsdorfer Allergie-Zentrum, Vienna. Additional sera were obtained from the Department of Dermatology, University Hospital, Zurich, Switzerland, and the Center for Clinical and Experimental Allergology, IDI-IRCCS, Rome, Italy. Samples were taken from a group of patients aged between 4 and 57 years with a typical case history of immediate hypersensitivity reactions to *Cladosporium herbarum*, a positive skin prick test to commercial *C. herbarum* extract or mold mix and a *C. herbarum* RAST class greater than 3. Sera from a non-allergic subject as well as a birch-allergic individual non-allergic to *Cladosporium herbarum* were used as negative controls.

2.2. Reagents

Unless otherwise stated, all chemicals used were obtained from Applichem GmbH, Darmstadt, Germany, via VWR International GmbH, Vienna, Austria. Enzymes were acquired from Fermentas Life Sciences GmbH, St. Leon-Rot, Germany, columns and chromatography media from GE Healthcare, Uppsala, Sweden. Primers were synthesized by MWG-Biotech AG, Ebersberg, Germany. Plasticware was purchased from Greiner Holding AG, Kremsmünster, Austria.

2.3. Construction of a *C. herbarum* cDNA expression library

As described previously (Achatz et al., 1995), a cDNA expression library was constructed in the Uni ZAP[®] XR vector (Stratagene, La Jolla, CA, USA) according to the manufac-

turer's instructions with poly A⁺-RNA isolated from the total *C. herbarum* RNA pool.

2.4. Screening of a *C. herbarum* cDNA library with human IgE antibodies

The library with a titre of 1.58×10^9 pfu/ml was screened using a serum pool from three *C. herbarum* allergic individuals recognizing allergens of the respective mold in the molecular weight range between approximately 20 and 30 kDa as determined by immunoblotting using a crude protein extract. About 6×10^5 phages were used to infect *E. coli* XL-1 blue cells. The latter were plated on LB agar and incubated at 37 °C overnight. Nitrocellulose filters (PROTRAN®, 0.45 µm, 132 mm diameter, Schleicher & Schüll, Dassel, Germany), pre-soaked with 10 mM IPTG, were layered on them and incubated at 41 °C for 4.5 h to induce protein synthesis. Filters were then lifted and blocked with "Gold Buffer" (40 mM Na₂HPO₄, 7 mM NaH₂PO₄, 0.5% BSA and 0.5% Tween-20) for 45 min under continuous agitation. After overnight incubation with the serum mixture mentioned above (diluted 1:10 in Gold Buffer), positive IgE-binding plaques could be visualized using ¹²⁵I-labeled anti-human-IgE-antibodies (5 µCi/5 ml, MedPro, Vienna, Austria) after a 1-week exposure to Hyperfilm™ (GE Healthcare, Uppsala, Sweden). Positive IgE-binding plaques were picked with a Pasteur pipette. Pure phages were treated according to the *in vivo* excision protocol to subclone their cDNA inserts into pBluescript SK- (Stratagene) via an ExAssist helper phage.

2.5. Screening of a *C. herbarum* cDNA library with radioactively labeled DNA probes

To exclude already identified *C. herbarum* allergens by hybridization screening, *E. coli* SOLR cells carrying the respective *in vivo* excised pBluescript SK-plasmid constructs were grown on LB-amp plates. To lift colonies, nitrocellulose filters were layered on top of the plates. Plasmid DNA was denatured for 5 min with 1.5 M NaCl/0.5 M NaOH and neutralized with 1 M Tris-HCl (pH 7.4)/1.5 M NaCl for further 5 min. Filters were rinsed with 2× SSPE (300 mM NaCl, 20 mM sodium phosphate, pH 7.0, 2 mM EDTA). Colonies were fixed by baking the filters for 2 h at 80 °C. Prehybridisation in buffer containing 100 mM sodium phosphate (pH 7.0), 850 mM NaCl, 2.5 mM EDTA, 0.1% SDS, 10× Denhardt's solution (0.2% BSA, 0.2% polyvinylpyrrolidone and 2% Ficoll) as well as 100 µg/ml salmon sperm DNA was performed for 1 h at 60 °C prior to overnight hybridisation with randomly [α -³²P]CTP-labeled PCR-fragments of the already known *C. herbarum* allergens Cla h 6 (enolase, recognized by 20% of the patients (Achatz et al., 1995)), Cla h 10 (aldehyde dehydrogenase, 36% (Achatz et al., 1995)), Cla h 7 (YCP4-homolog, 22% (Achatz et al., 1995)) and Cla h 5 (acidic ribosomal phosphoprotein P2, 22% (Achatz et al., 1995)) prepared using the Prime-a-Gene® Labeling System (Promega, Madison, WI, USA). High stringency washes were conducted with prewarmed

4× SSPE, 2× SSPE and 1× SSPE all containing 0.1% SDS for 30 min each. Filters were air-dried and again exposed to Hyperfilm™. Clones giving no signal to the four probes were further investigated because they represented potential new allergens.

2.6. DNA sequencing of the isolated cDNA inserts

Phagemid DNA from 4 ml *E. coli* SOLR overnight cultures was isolated by standard plasmid mini-preparation (GFX MicroPlasmid Preparation kit, GE Healthcare, Uppsala, Sweden) and sequenced completely on both strands using vector-derived forward T3 and reverse T7 primers by the ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Vienna, Austria). Homology searches and sequence comparisons of the cDNA- and deduced amino acid-sequences were performed through the National Center for Biotechnology Information using the basic local alignment search tool (BLAST, <http://www.ncbi.nlm.nih.gov/blast/>) as well as by comparison to the European Molecular Biology Laboratory (EMBL, <http://www.ebi.ac.uk/embl/>) and SWISS-PROT (<http://www.expasy.org/sprot/>) databases. Protein parameters such as amino acid composition, molecular weight and theoretical isoelectric point were calculated with the ProtParam tool accessible via the ExPASy proteomic server.

2.7. *C. herbarum* TCTP encoding cDNA: cloning into the expression vectors pHIS and pGST parallel 2

The open reading frame encoding the putative new mold allergen was amplified from the original pBluescript SK-cDNA clone using gene-specific oligodesoxynucleotides: a 5' forward primer with a BamH I (underlined) restriction site in front of the start ATG (5'-CGGGATCCATGCTGATCTACAACGACAT-3', $T_M = 68.4$ °C), and a 3' reverse primer introducing a Stu I (underlined) restriction site (5'-AAGGCCTTTACACCTTG-GTGGACTT-3', $T_M = 64.5$ °C). The 510 bp amplification product was subcloned into appropriately restricted, dephosphorylated vectors pHIS parallel 2 as well as pGST parallel 2, encoding an N-terminal hexahistidine- or GST-tag, followed by spacer region with a TEV protease cleavage site and a common polylinker sequence (Sheffield et al., 1999), referred to as ClaTCTP-6xHIS and ClaTCTP-GST, respectively.

2.8. Human TCTP encoding cDNA: cloning into the expression vectors pHIS and pGST parallel 2

The human TCTP homolog (named HRF or hTCTP, clone ID: IMAGp998F221621Q3) was obtained from the RZPD (Deutsches Ressourcenzentrum für Genomforschung GmbH, Berlin, Germany) in the vector pBluescript SK-. Its open reading frame was amplified by PCR using specific oligodesoxynucleotides containing a restriction site for BamH I (underlined) next to the ATG start codon for the 5' forward (5'-CGGGATCCATGATTATCTACCGGGACCTCAT-3', $T_M = 70.1$ °C) primer and a Stu I (underlined) restriction site

for the reverse primer (5'-AAGGCCTTTAACATTTTCCATT-TCTAAACCATCCTTAAAGAAATC-3', T_M = 67.4 °C). The PCR product was subcloned into the dephosphorylated vectors pHIS parallel 2 and pGST parallel 2 (Sheffield et al., 1999), referred to as hTCTP-6xHIS and hTCTP-GST.

2.9. Protein expression and recovery of the soluble supernatant

E. coli BL21 (DE3) cells transformed with *Cla*TCTP-6xHIS, *Cla*TCTP-GST, hTCTP-6xHIS or hTCTP-GST constructs were grown in LB medium containing 100 mg/l ampicillin at 37 °C until an OD₆₀₀ of 0.8. LacZ-promotor mediated protein expression of target genes was induced by the addition of 0.8 mM IPTG and cells were further grown for 20 h at 16 °C in order to enhance the formation of soluble recombinant fusion proteins. Cells were harvested by centrifugation and the pellet was resuspended in 1/50 volume of buffer recommended for the two different affinity matrices used. Starting buffer containing 50 mM Na₂HPO₄ (pH 8.0), 300 mM NaCl and 10 mM imidazole for Chelating Sepharose® Fast Flow affinity chromatography and GST-binding buffer (pH 7.3) including 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄ for the glutathione Sepharose 4B columns. The recombinant *Cla*TCTP-6xHIS, *Cla*TCTP-GST, hTCTP-6xHIS and hTCTP-GST fusion proteins were subsequently isolated from the cells by addition of 1 mg/ml lysozyme and three consecutive cycles of freezing and thawing prior to shearing the genomic DNA by ultrasonication.

2.10. Purification of *C. herbarum* TCTP and human TCTP via Ni²⁺ affinity chromatography

For purification by immobilised metal affinity chromatography (Chelating Sepharose® Fast Flow) under native conditions, the cleared lysate was directly loaded on a column equilibrated with Starting Buffer. Gradient elution was carried out from 100 to 500 mM imidazole in Starting Buffer under gravity flow. The purity of the fusion protein was controlled on Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA) stained 13.5% SDS-PAGE gels. After dialysis against 20 mM phosphate buffer (pH 7.4), the elution product was then used for cleavage of its HIS₆ fusion part using recombinant TEV protease (AcTEV™ protease, 10 U/μl, Invitrogen, Karlsruhe, Germany). Digestion was allowed to proceed at 29 °C for 4 h and at 4 °C overnight with 1 unit enzyme per 6 μg protein, followed by a second passage over a metal affinity column. TEV protease recognizes the seven amino acid sequence Glu-Asn-Leu-Tyr-Phe-Gln-Gly on the vector and cuts between glutamine and glycine so that only five vector-encoded amino acids remain at the N-terminus of the recombinant non-fusion protein (Mohanty et al., 2003), referred to as rnf-*Cla*TCTP and rnf-hTCTP. Homogeneity of the eluted fractions was evaluated by SDS-PAGE followed by silver-staining. Protein concentration was measured according to Bradford (Bradford, 1976) using a commercial kit (Bio-Rad Laboratories).

2.11. Purification of GST-tagged *C. herbarum* TCTP and human TCTP

Recombinant *Cla*TCTP-GST and hTCTP-GST fusion proteins were isolated from the bacterial lysates by binding to 1 ml glutathione-Sepharose 4B columns according to the manufacturer's protocol. The proteins were recovered from the matrix under mild non-denaturing conditions in elution buffer composed of 50 mM Tris-HCl (pH 8.0) and 10 mM reduced glutathione, thus preserving full antigenicity. Samples were analyzed by 13.5% SDS-PAGE gels.

2.12. Immunoblot analysis of *C. herbarum* TCTP and human TCTP

Immunoblot analyses with 50 μg of rnf-*Cla*TCTP and the same amount of rnf-hTCTP were performed as described previously (Simon-Nobbe et al., 2006). Briefly, membrane strips were probed with different sera diluted in Gold Buffer 1:10 overnight at 4 °C. We used 44 preselected patients' sera recognizing *C. herbarum* protein extract bands in the molecular weight range between 20 and 30 kDa as well as 108 further sera that were reactive to *C. herbarum* but not preselected in any other way. In total 306 sera reacting with *C. herbarum* protein extracts were available. The blots were incubated with ¹²⁵I-labeled anti-human IgE antibodies (1 μCi/ml, MedPro, Vienna, Austria). Binding of patients' IgE to the allergen was detected by scanning imaging plates (FujiFilm BAS Cassette 2325) with a Fujifilm BAS-1800 II instrument using the BAS-Reader software for Windows (Raytest, Straubenhardt, Germany) after 2 days of exposure. For testing of the competitive IgE binding to *C. herbarum* TCTP by inhibition blot analysis, positive sera were preincubated overnight at 4 °C with 100 μg rnf-hTCTP and then used to probe the PVDF membrane strips with blotted rnf-*Cla*TCTP as described above. Conversely, sera were preincubated with 100 μg rnf-*Cla*TCTP and then used to probe membrane strips with blotted rnf-hTCTP.

2.13. Enzyme-linked immunosorbent assay (ELISA) and inhibition-ELISA

0.5 μg of the purified GST-tagged proteins were bound to the surface of a microwell plate via a covalently coupled glutathione (Immobilizer™-glutathione plates, Nunc, Roskilde, Denmark). As reported previously (Schneider et al., 2006), the wells were incubated with 100 μl patients' sera diluted 1:5 in PBS-Tween containing 0.5% BSA (PBS-TB) prior to incubation with 100 μl horseradish peroxidase-conjugated goat anti-human IgE (Bethyl Laboratories, Montgomery, TX, USA) diluted 1:400 in PBS-TB. IgE was detected with 100 μl ABTS diammonium salt (0.22 mg/ml) in 0.05 M citric acid (pH 4.0) containing 0.5% H₂O₂. Specific binding of IgE antibodies was detected by measuring the absorption at 405 nm with a Spectra microplate reader (SLT Lab Instruments, Salzburg, Austria) and the absorbance in control wells was subtracted. For cross-inhibition experiments, 100 μl of sera diluted 1:5 in PBS-TB were preincubated with different amounts (10, 5, 1 and 0.5 μg) of rnf-*Cla*TCTP

or rnf-hTCTP overnight under constant shaking. The inhibition mixtures (including sera with no inhibitor as positive controls) were transferred to the washed wells coated with 0.5 µg of GST-tagged TCTP to proceed as mentioned above. The remaining TCTP-specific IgE antibodies were then measured using Delta-Soft Microplate Analysis Software. Negative controls included normal human serum (NHS) instead of the TCTP-specific antibodies as well as HRP-conjugated goat anti human IgE (second antibody) alone.

2.14. Histamine-releasing capacity of *C. herbarum* TCTP and human TCTP

For histamine-release studies, rnf-*Cla*TCTP, *Cla*TCTP-GST, rnf-hTCTP, hTCTP-GST as well as recombinant HRF (100% sequence identity to the hTCTP-GST described above) which was cloned as a GST-fusion in the baculovirus vector pVL1393 and purified from insect cells (Bheekha-Escura et al., 1999; Vonakis et al., 2001) were dialyzed against physiologic 1× PIPES buffer (pH 7.4) containing 25 mM PIPES, 110 mM NaCl and 5 mM KCl. Peripheral blood was obtained from patients by venipuncture after informed consent. Basophils were isolated from heparinized blood samples by a standard dextran sedimentation method as described previously (Schleimer et al., 1982) and finally challenged with different amounts of protein for 45 min at 37 °C. Samples were then centrifuged and the supernatant was analyzed for histamine content by means of an automated fluorometric method as described elsewhere (Siraganian, 1975). The results based on duplicate determinations were expressed as a percentage (relative to total histamine) of histamine release after subtracting the spontaneous release of unstimulated cells determined in control samples containing buffer only.

2.15. Far-UV circular dichroism spectropolarimetry

Circular dichroic spectra of rnf-*Cla*TCTP and rnf-hTCTP in the range between 190 and 260 nm wavelength were recorded in 10 mM sodium phosphate buffer (pH 7.4) with a J-810 spectropolarimeter (JASCO Inc., Easton, MD, USA) in continuous scanning mode using a 1.0 mm path length quartz cuvette, a sensitivity of 100 mdeg, a resolution of 1 nm, a scanning speed of 100 nm/min, a response of 1 s and a bandwidth of 1 nm. The baseline obtained with pure buffer in the absence of protein was subtracted from the sample spectrum and each circular dichroic profile represents an average of 5 scans. Data were expressed as mean residue molar ellipticity.

3. Results

3.1. Identification and isolation of a *C. herbarum* TCTP-encoding cDNA clone

Many *C. herbarum* allergic patients show strong IgE reactivity to proteins in the molecular weight range between 18 and 30 kDa, but only the two allergens mannitol dehydrogenase (*Cla* h 8, AY191816, 29 kDa) and YCP4 homolog (*Cla* h 7, X78224,

22 kDa) have so far been cloned and characterized in this size interval (Achatz et al., 1995; Simon-Nobbe et al., 2006).

Forty-two plaque-purified clones were isolated by IgE screening of a newly constructed *C. herbarum* cDNA expression library (see Section 2). We tested for hybridization of the cDNA clones with probes corresponding to the known allergens enolase, aldehyde dehydrogenase, YCP4 homolog and acidic ribosomal phosphoprotein P2, thereby reducing the number to 19 clones possibly encoding new allergens. After *in vivo* excision of these clones, phagemid DNAs were isolated, sequenced and the deduced amino acid sequences further analyzed by NCBI BLAST searches. Two of the 19 clone inserts were shown by DNA sequencing to code for a new *C. herbarum* allergen highly homologous to human TCTP (histamine releasing factor).

3.2. DNA sequencing of *C. herbarum* TCTP and data analysis

The two TCTP-homologous clones contained identical open reading frames spanning 510 base pairs, beginning with a methionine at position 45 and ending with a TAA termination codon at nucleotide position 552 (predicting a protein of 169 amino acids) as well as the 5' untranslated region (44 nucleotides) and the polyadenylated 3' tail (226 nucleotides) as illustrated in Fig. 1. The complete cDNA sequence was deposited in the National Center for Biotechnology Information (NCBI) GenBank database with the accession number AY349609. Analysis of the deduced amino acid sequence with the BLASTP algorithm showed the highest similarity to *Saccharomyces cerevisiae* TCTP (accession number NP_012867, systematic name YKL056c, 55% identical and 75% similar amino acids) and to *Schizosaccharomyces pombe* TCTP (accession number: NP_594328, systematic name p23fy, 53% identical and 73% similar amino acids), which in turn display strong similarity to human translationally controlled tumor protein, also described as histamine releasing factor. A detailed comparison between *Cladosporium herbarum* TCTP, human TCTP and other selected eukaryotic homologous proteins by multiple sequence alignment (<http://www.ebi.ac.uk/clustalw>) is presented in Fig. 2. 70 out of 169 amino acids are identical between the human and the fungal protein reflecting a sequence identity of 42% and an overall similarity of 57% (96 out of 169 amino acids). *Cladosporium herbarum* TCTP with 19 predominantly basic (K, R), 33 strongly acidic (D, E), 55 hydrophobic (A, I, L, F, W, V) and 37 primarily polar (N, C, Q, S, T, Y) amino acids has a calculated molecular mass of 18.7 kDa and an isoelectric point of 4.43. It possesses 14 residues (M₁, E₁₂, D₁₆, N₄₇, S₄₉, E₅₁, V₆₈, L₇₃, K₈₀, K₈₈, F₁₁₁, G₁₃₄, P₁₅₅, K₁₆₈) completely conserved across the whole TCTP family, suggesting a participation in physiologic functions. Serines 9, 49, 97, 101, 104, 125 and 166, threonine 154 as well as tyrosines 4 and 18 represent potential phosphorylation sites as analyzed using the NetPhos 2.0 Program provided by the Technical University of Denmark (<http://www.cbs.dtu.dk/services/NetPhos/>). Further studies on the NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) demonstrated that N₄₇–A₄₈–S₄₉ represented a putative N-glycosylation motif con-

5'	CAGCATCTTACACTCGTTCCACACTCTCCACAATACCGCCAAC	44
ATG CTG ATC TAC AAC GAC ATC ATC TCC GGT GAC GAG	80	
M L I Y N D I I S G D E		
CTC ATC TCC GAC TCG TAC GAC CTT GTT GAG GTT GAC	116	
L I S D S Y D L V E V D		
GGT GTC GCC TAC GAG GCC GAC TGC CGC AAG ATC ACC	152	
G V A Y E A D C R K I T		
ATC GGT GGT GAG ACC TTC GAC ACT GGC GCC AAC GCT	188	
I G G E T F D T G A N A		
TCC GCC GAG GGC GGC GAT GAG GAG GCC GAC GAC CAG	224	
S A E G G D E E A D D Q		
AAG GAG ACC AAG ATC GAC GTC GTC CAC GCT TTC CAG	260	
K E T K I D V V H A F Q		
CTC CAG GAG ACC AAC TTC GAC AAG AAG GCA TAC CTC	296	
L Q E T N F D K K A Y L		
GGC CAC CTC AAG TCT TAC ATG AAG AAG ATC AAG GAG	332	
G H L K S Y M K K I K E		
TCC ATG GCT GCC AGC GGC GCC AGC GAG GAT GAG GTC	368	
S M A A S G A S E D E V		
AAG GAG TTC GAG AAG GGT GCT CAG ACT TTC GCC AAG	404	
K E F E K G A Q T F A K		
AGG GTC GTC GGC AGC TTC AAG GAC TAC GAG TTC CTG	440	
R V V G S F K D Y E F L		
ATC GGC CCC TCC ATG GAC CCC GAT GCC ATG GTT GTT	476	
I G P S M D P D A M V V		
CTC CTC AAC TAC CGC GAG GAC GGT GTC ACC CCT TAC	512	
L L N Y R E D G V T P Y		
GTT ACT CTG TGG AAG CAC GGC CTC AAG TCC ACC AAG	548	
V T L W K H G L K S T K		
GTG TAA	554	
V *		
GCGATTTTGGCAAAATGTTGAGCGAAGAGGTGCAAGGCAGGAGCTC	599	
TGAAGCGTTGACGAAGCTGCTCTCGAGCTCTTTGCTACGAACGATAT	644	
CCCAGGACGAGCATTATGGTAGACATGATTTTGGAAATAACCTCAGG	689	
CTCTGCTTGAGTCTTTCCACCCAAAAGTTGAAACAACTTCCATCC	734	
CATGAAAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAAAG	780	
	3'	

Fig. 1. DNA- and deduced amino acid sequence of the isolated full-length *Cladosporium herbarum* TCTP clone with an estimated molecular mass of 18.7 kD. The nucleotide sequence represents the whole pBluescript EcoR I/Xho I cDNA insert. The amino acid-sequence begins at the first potential in-frame initiation ATG and ends with the TAA stop codon as denoted by the asterisk, followed by the 3' untranslated region and the polyadenylated tail. Numbers on the right indicate the nucleotide positions. Amino acid regions residing in the commonly conserved TCTP1 and TCTP2 signature patterns are boxed.

served among several species, although intracellular proteins lacking any signal sequences are unlikely to undergo such a posttranslational modification *in vivo*.

A search of our *C. herbarum* TCTP protein sequence with the pattern database of PROSITE (<http://www.expasy.ch/prosite/>) revealed that excluding threonine 44, the amino acid positions 45–54 correspond to the well-conserved TCTP1 signature sequence [IFAE]–[GA]–[GAS]–N–[PAK]–S–[GTA]–E–[GD-EV]–[PAGEQV]–[DEQGAV] as marked in Fig. 1. However, it only shows fragments of a typical TCTP2 region [FLIV]– $x_{(4)}$ –[FLVH]–[FY]–[MIVCT]–G–E– $x_{(4)}$ –[DENP]–[GAST]– x –[LIVM]–[GAVI]– $x_{(3)}$ –[FYWQ] found in all of the known translationally controlled tumor proteins except the *Hydra vulgaris* and *Schistosoma mansoni* TCTPs (Rao et al., 2002; Yan et al., 2000). The sequence-based secondary structure of *C. herbarum* TCTP as well as a three-dimensional model were computed with the Prediction Server PSIPRED

(<http://bioinf.cs.ucl.ac.uk/psipred/psiform.html>) and the online available program 3D-JIGSAW (<http://www.bmm.icnet.uk/servers/3djigsaw/>), respectively, exhibiting significant similarity to *Schizosaccharomyces pombe* TCTP (Fig. 3). As recently determined by nuclear magnetic resonance spectroscopy, the structure of the latter displays a relationship to the human protein Mss4, which is a guanine nucleotide-free chaperone of the Rab protein implicated in vesicle transport and intracellular trafficking (Thaw et al., 2001).

3.3. Expression and purification of *C. herbarum* TCTP and human TCTP

The complete cDNA sequences encoding *C. herbarum* TCTP as well as human TCTP were expressed in *E. coli* as hexahistidine-tagged fusion proteins and purified by Ni^{2+} chelate affinity chromatography under non-denaturing condi-

<i>C. herbarum</i>	MLIYNDIISGDELISDSYDVEVDG-VAYEADCRKITIGGETFD---TGANASAE-GGDEE	56
yeast	MIIYKDIIFSNDLLSDYDAKLVDV-VIYEADCAAMNVGCDNID---IGANPSAE-GGDD	56
human	MIIYEDLISHDEMFSDIWKIREIADGLCLEVEGKMVSRTGNIDSLIGGNASAE-GPEGE	60
mouse	MIIYEDLISHDELFSDIWKIREIADGLCLEVEGKMVSRTGAIDSLIGGNASAE-GPEGE	60
<i>Arabidopsis</i>	MLVYCDLLTGDELLSDSPYKEIENGILWEVEGKVVTVGAVDVN---IGANPSAEEGGEDE	58
Alfalfa	MLVYCDLLTGDELLSDSPYKEIENGMLWEVEGKVVTKGVVEVD---IGANASAE-GGEDE	57
<i>C. herbarum</i>	-ADDOKETKIDVWHAFQLQET-NFDKKAYLGHLSYMKKIKESMAASGASEDEVKEFEKGA	114
yeast	-VEEGAEMVNVVHVSFRLQET-AFDKKSFLTYIKCYMKAVKAKLQETNP---EEVPKFEKGA	112
human	GTESITVITGVDIVMNHHLQET-SFTKEAYKRYIKDYMKSLKGLKEEQRP---ERVKPFMTGA	117
mouse	GTESITVITGVDIVMNHHLQET-SFTKEAYKRYIKDYMKSLKGLKEEQRP---ERVKPFMTGA	117
<i>Arabidopsis</i>	GVDDSTQKVVIVDTFRLQEQFTYDKKGF IAYIKRYIKLLTPKLSEEDQ-----AVFKKGI	113
Alfalfa	GVDDTAVKVVIVDVFRQLQEQAFDKKQFLGFVKRYIKLLTPKLDAEKQ-----ELFKKHI	112
<i>C. herbarum</i>	QTFAKRWVGSFKDYEFLLGPSMDPDAMVVLNLYREDGVTPYVTLUKHGLKSTKV	169
yeast	QTYVKVIGSFKDWFFIGESMDPDAMVVLNLYREDGTPFVAIVKHGIVEEKI	167
human	AEQIKHILANFNKYOFFIGEMNPDGMVALLDYREDGVTPYMIFFKDGLEMEKC	172
mouse	AEQIKHILANFNKYOFFIGEMNPDGMVALLDYREDGVTPYMIFFKDGLEMEKC	172
<i>Arabidopsis</i>	EGATRFLLPRLSDFQFFVGEGLHDDSTLVFAFYKEGSTNFTFLYFAHGLKEVKC	168
Alfalfa	EGATRYLLCKLKDLOFFVGESLHDDGSLVFAFYKDGADFTFLYFAALKEIKC	167

Fig. 2. ClustalW alignment (<http://www.ebi.ac.uk/clustalw/>) of several amino acid sequences of the TCTP family of proteins, showing a high degree of homology between phylogenetically distinct members: *Cladosporium herbarum* (accession no. AY349609), *Saccharomyces cerevisiae* (YKL056c, accession no. NP_012867), *Homo sapiens* (p23, accession no. NP_003286), *Mus musculus* (p21, accession no. NP_033455), *Arabidopsis thaliana* (accession no. NP_188286) and alfalfa (accession no. P28014). Numbers indicate the amino acid residues (without gaps), starting at the NH2-terminal methionine. Identical amino acid residues in at least three of the sequences are highlighted in black, similar ones in grey. The identity between *C. herbarum* and the human TCTP is approximately 41%.

tions resulting in yields of 27.5 and 22.1 mg pure recombinant protein per litre culture, respectively. The fusion proteins, the majority of which was present in the fraction eluted with 100 mM imidazole in both cases, appeared as prominent bands on Coomassie-stained SDS-PAGE gels as illustrated in Fig. 4. However, their apparent molecular weights are considerably larger than the calculated ones, as has been noticed before

(Sanchez et al., 1997). Subsequently, the fusion tag of both *Cla*TCTP-6xHIS and hTCTP-6xHIS was cleaved by recombinant TEV protease and samples were subjected to a second affinity chromatographic step. The purified recombinant non-fusion (rnf) proteins were found to be essentially depleted from *E. coli* contaminants as determined by denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis and Coomassie

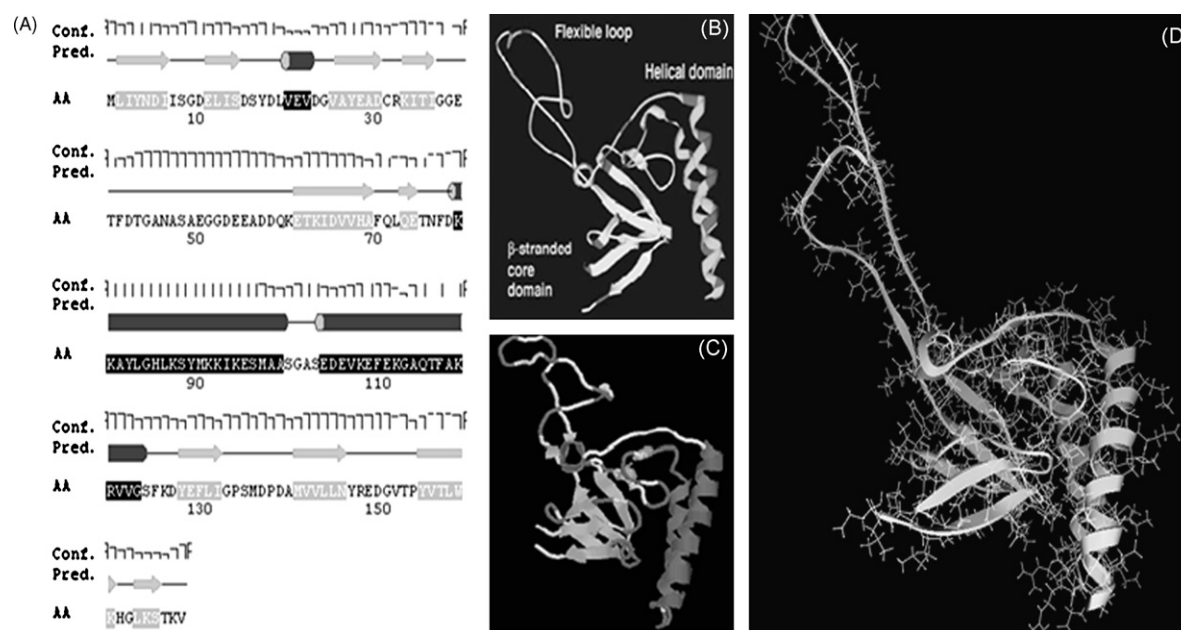


Fig. 3. (A) Computed secondary structure elements of *Cladosporium herbarum* TCTP (<http://bioinf.cs.ucl.ac.uk/psipred/psiform.html>), indicating significant similarity to its fission yeast homolog. Lines represent coiled regions, grey arrows indicate β-strands and black cylinders symbolize helices. Conf.: confidence interval (0 = low, 9 = high degree of probability), Pred.: graphical illustration of the predicted secondary structure, AA: *C. herbarum* TCTP amino acid sequence. (B) Three-dimensional model for *S. pombe* TCTP (Bommer and Thiele, 2004) and (C) a predicted *C. herbarum* structure modelled using 3D Jigsaw. (D) Solution structure of human translationally controlled tumor protein as determined by NMR (www.rcsb.org/pdb, pdb code: 2HR9).

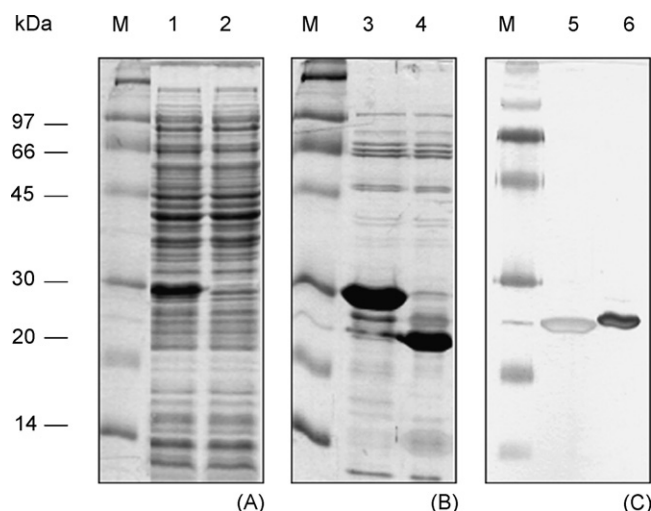


Fig. 4. Expression and purification of *C. herbarum* TCTP. (A) Proteins extracted from IPTG-induced (lane 1) and uninduced (lane 2) cultures of *E. coli* BL21 (DE3) transformed with *Cla*TCTP-6xHIS expression constructs were separated on a 13.5% SDS-PAGE gel under reducing conditions and Coomassie-stained. (B) *C. herbarum* TCTP was then purified from the cultures by Ni^{2+} affinity chromatography (lane 3) prior to cleavage of the fusion tag by TEV protease digestion (lane 4). (C) Silver staining of 5 μg rnf-*Cla*TCTP (lane 5) and the appropriately purified rnf-hTCTP (lane 6) after elimination of co-eluting Ni^{2+} -binding impurities by enzymatical digestion of the HIS₆-tag followed by a second passage over the affinity column. Molecular mass standards (M) are indicated on the left side.

as well as silver staining (Fig. 4), respectively, and their CD spectra were almost superimposable and indicative of a well-folded protein (Fig. 5).

Both *C. herbarum* and human TCTP were also expressed as fusion proteins with *Schistosoma japonicum* glutathione S-transferase and purified via Glutathione Sepharose 4B columns (yielding 3.1 and 3.6 mg pure recombinant affinity-tagged protein per litre culture), resulting in a single band around 45 kDa (18.7 kDa for *Cla*TCTP or 19.6 kDa for its human homolog plus 26 kDa for the GST-tag) on a 13.5% SDS-PAGE gel (Fig. 6). Although the 211 amino acid fusion part is larger than the in-frame encoded TCTP itself, the GST-tag does not interfere with the protein's immunological characteristics as was demonstrated for the HRF-GST (Schroeder et al., 1996).

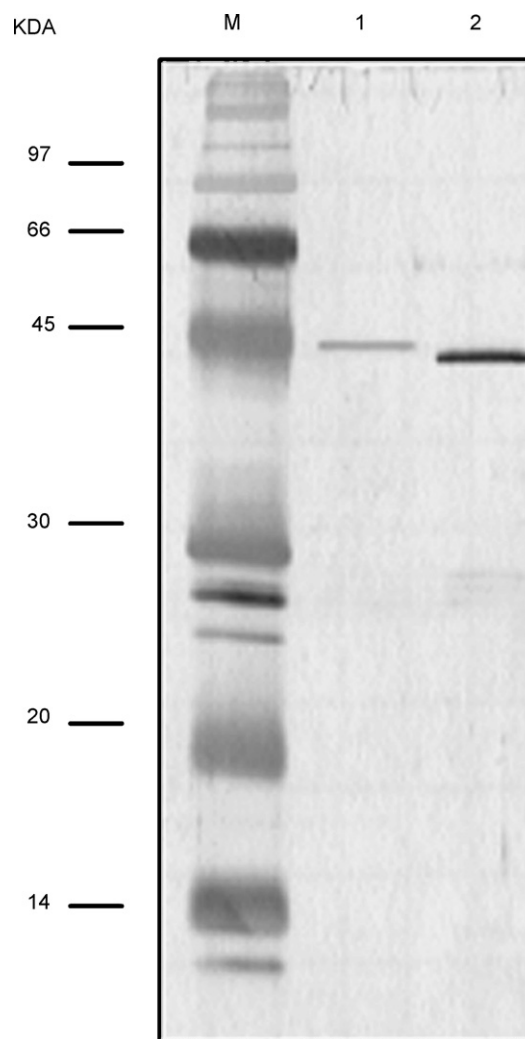


Fig. 6. Silver staining of 1 μg *Cla*TCTP-GST (lane 1) and hTCTP-GST (lane 2) purified over glutathione-coupled Sepharose 4b columns. Molecular mass standards (high molecular weight rainbow marker, M) in kDa are shown on the left side.

3.4. Immunoreactivity of *C. herbarum* TCTP and cross-reactivity with human TCTP

The importance of *C. herbarum* TCTP as an allergen was assessed by subjecting 50 μg of rnf-*Cla*TCTP as well as rnf-hTCTP to immunoblot analysis. The ability of our newly identified protein to bind IgE from nine patients in immunoblot analyses and ELISA assays provides strong evidence for its allergenicity (Fig. 7A). The prevalence of IgE reactivity to *Cla*TCTP is approximately 3% as 9 out of 306 *C. herbarum* reactive patients reacted to the recombinant protein. Thus, *Cla*TCTP is a minor allergen. However, five of the nine individuals showing the strongest signals to *Cla*TCTP were also recognizing its human homolog (Fig. 7B).

We further investigated whether *Cla*TCTP and hTCTP actually share common IgE-binding cross-reactive epitopes by inhibition immunoblots as well as by ELISA assays. Whereas serum of patient 1 preabsorbed with 100 μg rnf-hTCTP did not obviously reduce IgE binding to *Cla*TCTP in the first case, the

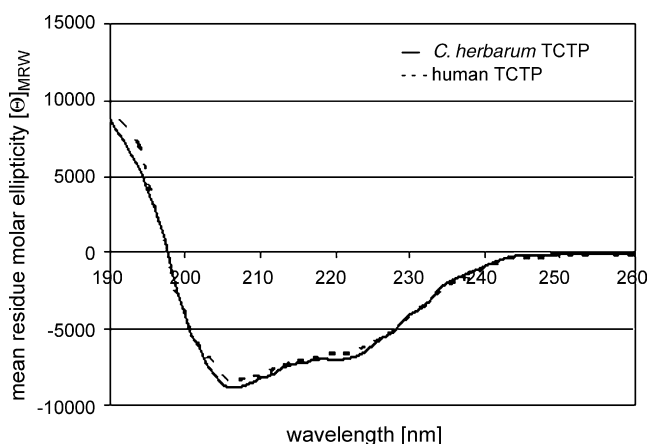


Fig. 5. Circular dichroism spectra of rnf-*Cla*TCTP and rnf-hTCTP.

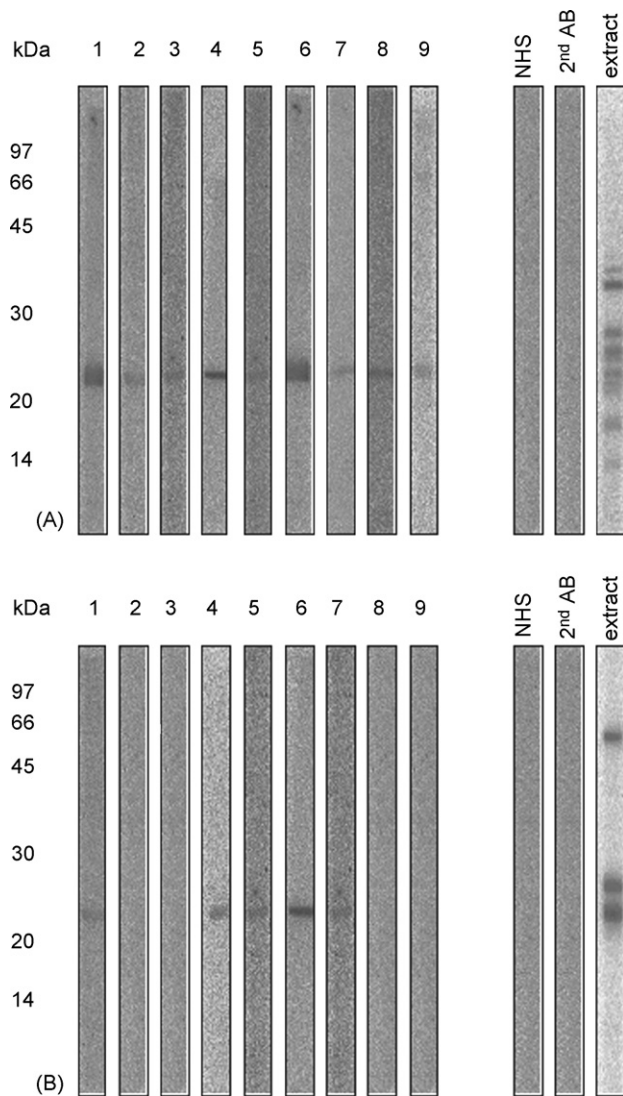


Fig. 7. Immunoblot analysis for specific IgE antibodies against rnf-*Cla*TCTP and rnf-hTCTP. (A) Nine out of 306 individuals with a positive RAST to *C. herbarum* recognize rnf-*Cla*TCTP. (B) Five of these patients (1, 4, 5, 6 and 7, but not subjects 2, 3, 8 and 9) also react with its human homolog, thereby indicating significant cross-reactivity between these two proteins. Negative control experiments included serum of a non-atopic individual (NHS) and usage of second antibody only (^{125} I-labeled-rabbit-anti-human-IgE-antibody). As a positive control the immunoreactivity of patients 2 (A) and 9 (B) against the *C. herbarum* crude protein extract is shown.

same serum preincubated with the equal amount of rnf-*Cla*TCTP could inhibit antibody binding to hTCTP (Fig. 8). A complete disappearance of the protein band could be observed, however, when rnf-*Cla*TCTP transferred to the PVDF membrane was tested with serum preincubated with rnf-*Cla*TCTP itself and *vice versa*. The obtained results suggest that the *C. herbarum* TCTP allergen apparently possesses more antigenic determinants than its human homolog and that patient 1 is primarily sensitized against the *C. herbarum* TCTP.

Similar findings were obtained by ELISA inhibition experiments. When *C. herbarum* TCTP was coated to the ELISA microwell plate and tested with aliquots of serum after preincubation with increasing amounts of rnf-hTCTP (0.5, 1, 5 and

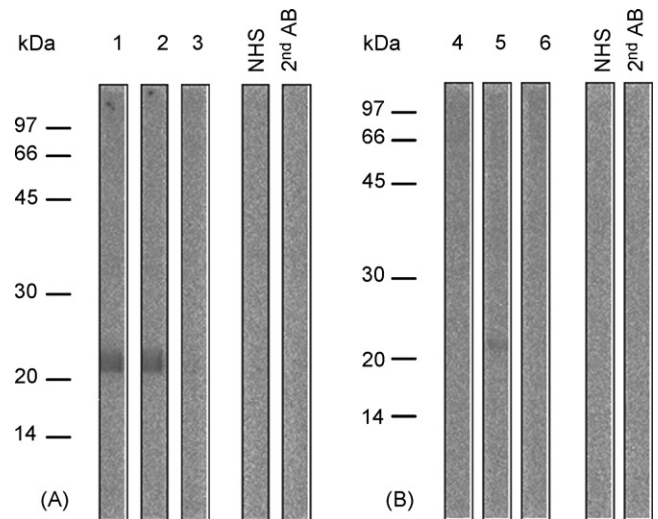


Fig. 8. Inhibition immunoblot to establish cross-reactivity between *C. herbarum* TCTP and human TCTP. (A) Rnf-*Cla*TCTP blotted on a PVDF membrane was probed with (1) serum of patient 1 preincubated with 100 μ g rnf-hTCTP, (2) serum without preincubation as a positive control and (3) serum preabsorbed with 100 μ g rnf-*Cla*TCTP itself. (B) Human TCTP transferred to a PVDF membrane was analysed using (4) the same serum preincubated with 100 μ g rnf-*Cla*TCTP, (5) serum without preincubation as indicated in Fig. 6 and (6) serum preabsorbed with 100 μ g rnf-hTCTP. Negative control experiments included serum of a non-atopic individual (NHS) and usage of second antibody only. Molecular mass standards in kDa are shown on the left side.

10 μ g) added to the fluid phase, the latter resulted in a substantial, dose-dependent reduction of the TCTP-specific signal (Fig. 9A). The reverse reaction was also performed with the effect that rnf-*Cla*TCTP was able to completely inhibit the binding of IgE antibodies to human TCTP (Fig. 9B). As expected, no signal could be detected when using the serum from one healthy person with negative case history of any type I allergy (NHS), and additional controls, in which the incubation with serum was omitted (second antibody only) or the serum was preabsorbed with the corresponding TCTP itself. Inhibition rates were finally calculated under consideration of the non-specific binding control after subtraction of the background activity measured with pure ABTS solution in uncoated wells that were neither incubated with serum nor with the second antibody. To summarize, human TCTP was able to diminish about 76.5% of IgE-binding at a concentration of 0.5 μ g and up to 85.7 and 88.9% when using between 5 and 10 μ g pure recombinant protein. On the other hand we observed a complete inhibition (100%) of IgE binding to human TCTP after preincubating the serum with 5 or 10 μ g rnf-*Cla*TCTP, whereas 98.2 and 97.5% reduction of antibody binding were observed with 1 and 0.5 μ g rnf-*Cla*TCTP.

To warrant minimal steric hindrance of TCTP epitope accessibility, we did not immobilize the protein to conventional ELISA microwell plates by passive adsorption, but attached purified GST-tagged *Cla*TCTP and hTCTP to a covalently coupled glutathione distanced from the polystyrene surface by a hydrophilic linker. Serum preincubation was performed with TEV-digested, recombinant non-fusion TCTP, however, to ensure comparability between the results obtained by Western blotting and inhibition-ELISA.

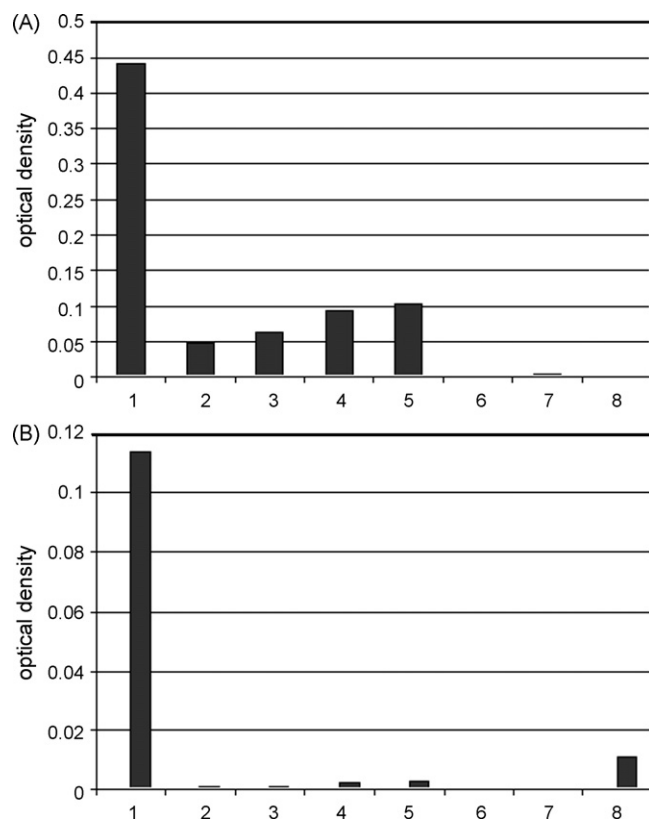


Fig. 9. Inhibition ELISA. (A) Serum samples obtained from patient 6 were preincubated with different amounts of rnf-hTCTP and transferred to wells coated with GST-tagged *C. herbarum* TCTP to measure bound IgE by antigen-specific enzyme-linked immunosorbent assay. Shown are (1) serum without preincubation, resulting in an optical density of 0.442, and serum preincubated with 10 μ g (2), 5 μ g (3), 1 μ g (4) and 0.5 μ g (5) rnf-hTCTP. Control wells included normal human serum NHS (6), serum of patient 6 preincubated with 10 μ g rnf-*Clat*TCTP itself (7) and second antibody only (8). (B) Serum samples obtained from patient 6 were preincubated with different amounts of rnf-*Clat*TCTP and transferred to wells coated with GST-tagged human TCTP. The numbering is identical as in (A), except that serum was preincubated with mold TCTP in lanes 2–5 and with 10 μ g rnf-hTCTP in position 7, respectively.

3.5. Histamine releasing potential of *C. herbarum* TCTP

Since mammalian TCTPs are known to cause degranulation of basophils, it was important to show that our *C. herbarum* TCTP was biologically active as a histamine-releasing factor as well. In order to confirm its biologic function, its ability to induce histamine release from dextran sedimented human basophils isolated from three severely asthmatic patients was measured. The achieved amount of mediator release of 15% at a protein concentration of 141 μ g/ml *Clat*TCTP-GST (data not shown) as well as of 8 and 25% at a protein concentration of 50 and 100 μ g/ml rnf-*Clat*TCTP as stimulus was approximately between 20 and 50% of the control release obtained with authentic purified rnf-hTCTP/hTCTP-GST or HRF-GST (Fig. 10, positive control). Analogous results indicating that the human TCTP was a stronger stimulus than the *C. herbarum* allergen and similar to HRF-GST were obtained in four other experiments (data not shown). Histamine measurements were performed in duplicate for each stimulus. These results show that

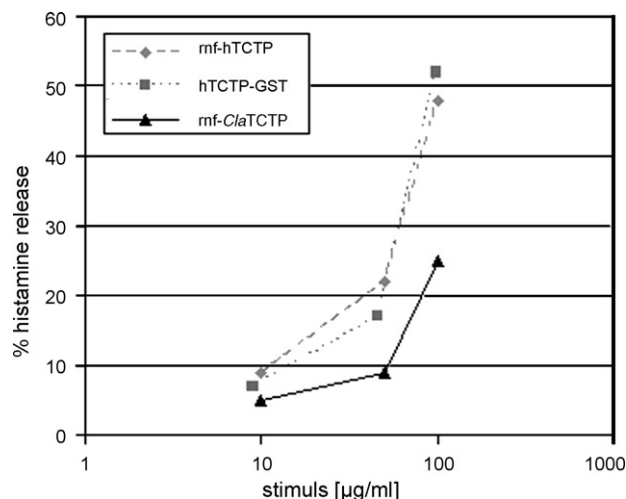


Fig. 10. Basophil histamine release induced with different amounts of rnf-*Clat*TCTP ($\blacktriangle$) and rnf-hTCTP ($\blacklozenge$). HRF-GST ($\blacksquare$) purified in the laboratory of MacDonald et al. served as an internal control. The x-axis displays the amount of stimulus (μ g/ml), the y-axis the %histamine release.

the new allergen of *C. herbarum*, *Clat*TCTP, displays significant *in vitro* histamine release activity with basophils obtained from severely asthmatic patients. The maximum amount of histamine release from basophils that was achieved with *Clat*TCTP was 24 ng/ml. It is interesting to note that none of the three severely allergic donors of basophils had IgE recognizing *Clat*TCTP or hTCTP (data not shown).

4. Discussion

Our data show that *Clat*TCTP, the *C. herbarum* ortholog of the multifunctional human protein TCTP or HRF, is a new allergen of the mold *C. herbarum*. This new allergen clearly is a minor allergen, because the incidence of IgE reactivity is 3% (9 of 306 patients) among allergics sensitized to *C. herbarum*. However, analyzing the clinical history of these patients reveals a possible strong association with severe asthma and with relatively high IgE levels in patients recognizing *Clat*TCTP. Seven of nine patients positive for *Clat*TCTP are asthmatics, while only about 20% display asthma in the remaining *C. herbarum* allergic population. Those five patients who show the strongest bands in IgE immunoblots of *Clat*TCTP are also the ones who show cross-reactivity with hTCTP. Cross inhibition experiments indicate that the patients were probably primarily immunized with the fungal protein and show only cross-reactivity and not co-sensitization to the human protein. This assumption is based on the fact that pre-incubation with *Clat*TCTP can completely inhibit binding to hTCTP, but not *vice versa* as was demonstrated in immunoblots as well as in inhibition ELISA experiments (Figs. 8 and 9). It has been shown that the majority of hTCTP/HRF is not dependent on the IgE molecule for its bioactivity which is despite its original terminology. Parenthetically, serum from human HRF/TCTP responders as defined by MacDonald and co-workers (Bheekha-Escura et al., 1999, 2000; Wantke et al., 1999) did not show cross-reactivity to *Clat*TCTP or hTCTP (data not shown). However, the *Clat*TCTP is rec-

ognized by a small but highly allergic, asthmatic subgroup of the patients allergic to the fungus. Therefore, in this subpopulation, *ClatTCTP* can function as an autoreactive IgE binding antigen.

Similar cases of auto-reactivity of fungal allergens to human proteins have been observed repeatedly (Cramer et al., 2006). In all cases, these proteins were highly conserved and abundant eukaryotic intracellular proteins, like MnSOD (Appenzeller et al., 1999; Schmid-Grendelmeier et al., 2005) and P2 acidic ribosomal phosphoprotein (Mayer et al., 1999). TCTP is also an abundant eukaryotic protein due to its involvement in the translation machinery. The structures of these highly conserved proteins were either determined experimentally or could be modeled *ab initio* with high confidence. In the case of *ClatTCTP* and hTCTP, prediction led to a structure highly similar to the homologous *S. pombe* structure determined by NMR (Fig. 3). Very probably, the whole family of eukaryotic TCTP proteins including hTCTP displays a similar three-domain structure and therefore also similar B-cell epitopes leading to the observed cross-reactivity. However, we go one step further and suggest that the above-mentioned association with the more severe cases of asthma could have a mechanistic basis in the observed cross-reactivity (Zhao et al., 1998). In the case of MnSOD and atopic dermatitis such a hypothesis could be substantiated (Schmid-Grendelmeier et al., 2005). The suggested pathomechanism consists of three important facts: (i) the severe form of disease is accompanied by chronic inflammation and cell lysis; (ii) MnSOD is overexpressed in the inflamed tissue as a reaction to oxidative stress; (iii) liberation of MnSOD in this scenario leads to binding of antibodies, aggravation of the disease, and in the long run to disease symptoms which are independent of the presence of allergen. It is possible, but by no means proven that a similar pathomechanism may take place in the lung, for instance in severe asthma. Clinical studies have shown repeatedly that sensitization to fungi, in particular *C. herbarum* and the closely related *A. alternata*, is an important risk factor for developing severe asthma (Black et al., 2000; Ezeamuzie et al., 2000; Zureik et al., 2002). The nine patients reactive to *ClatTCTP* are young adults, four female and five male predominantly multiply sensitized and aged 27.1 ± 8.16 (standard deviation) years. We speculate that they are at risk to develop severe allergen-independent asthma later in their life. We are presenting here this pathomechanism as an interesting but nevertheless speculative hypothesis. Open questions in this connection are: Do all or most of the severely sick asthmatics express HRF in serum? What happens to the HRF after binding of IgE? Will the young adults identified which react to TCTP later develop allergen-independent asthma? Is there a vicious cycle leading to more extracellular expression of TCTP (HRF) in the severely asthmatic patients?

TCTP (HRF) is a B-cell cytokine with a strong pro-inflammatory activity. This may be the reason why a whole number of human parasites secrete large amounts of “their” TCTP into the host, ultimately causing high fever (the Malaria parasite, *Plasmodium falciparum*) and granuloma (*Schistosoma mansoni*) (MacDonald et al., 2001). Incidentally, the same parasites also cause a strong IgE reactivity in the patient.

Most of our *C. herbarum* allergic patients were found to be simultaneously sensitized to several fungi and in addition to several other allergens, like pollen, animal dander and foodstuffs. The asthmatics sensitized to TCTP are no exception to this rule. Their allergic symptoms besides asthma are also mostly multiple including rhinitis, conjunctivitis, and occasionally atopic dermatitis.

Finally, we want to briefly discuss the importance of knowing the allergens of *C. herbarum* for medical research and for developing a rational therapy for the allergic patient. *C. herbarum* is one of the most frequently encountered airborne mold species worldwide. The incidence of hypersensitivity reactions against *C. herbarum* depends on the climate and varies between 3 and 30% of the allergic population (Cramer et al., 2006). In a large European multicenter report promoted by the Academy of Allergy and Clinical Immunology (D’Amato et al., 1997), about 10% of the patients with allergic nasal or bronchial symptoms were found sensitized to *Cladosporium* and/or *Alternaria*, with the lowest prevalence in Portugal (3%) and the highest in Spain (20%). These numbers most likely underestimate the prevalence of sensitization to *C. herbarum*, because the obtained results strongly depend on the commercial fungal extracts used in routine assessments and suffer from a low sensitivity due to strain variability, culture methods, origin of source materials and differences in extraction procedures, thus often generating false negative test outcomes (Breitenbach and Simon-Nobbe, 2002). Therefore, knowing the allergen complement of *C. herbarum* and being able to produce the allergens in a pure and standardized form for diagnosis and immune therapy is important. Additionally, some allergens, like the auto-reactive allergen described here can possibly help to understand the pathomechanism of severe asthma, as outlined above.

Acknowledgements

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References

- 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.
- 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.
- 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.

- 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.
- 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.
- Cans, C., Passer, B.J., Shalak, V., Nancy-Portebois, V., Crible, V., Amzallag, N., Allanic, D., Tufino, R., Argentini, 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.
- Crameri, R., Weichel, M., Fluckiger, S., Glaser, A.G., Rhyner, C., 2006. Fungal allergies: a yet unsolved problem. *Chem. Immunol. Allergy* 91, 121–133.
- D'Amato, G., Chatzigeorgiou, G., Corsico, R., Gioulekas, D., Jager, L., Jager, S., Kontou-Fili, K., Kouridakis, S., Liccardi, G., Meriggi, A., Palma-Carlos, A., Palma-Carlos, M.L., Pagan Aleman, A., Parmiani, S., Puccinelli, P., Russo, M., Spijksma, F.T., Torricelli, R., Wuthrich, B., 1997. Evaluation of the prevalence of skin prick test positivity to *Alternaria* and *Cladosporium* in patients with suspected respiratory allergy. A European multicenter study promoted by the Subcommittee on Aerobiology and Environmental Aspects of Inhalant Allergens of the European Academy of Allergy and Clinical Immunology. *Allergy* 52, 711–716.
- Ezeamuzie, C.I., Al-Ali, S., Khan, M., Hijazi, Z., Dowaisan, A., Thomson, M.S., Georgi, J., 2000. IgE-mediated sensitization to mould allergens among patients with allergic respiratory diseases in a desert environment. *Int. Arch. Allergy Immunol.* 121, 300–307.
- Fleischer, T.C., Weaver, C.M., McAfee, K.J., Jennings, J.L., Link, A.J., 2006. Systematic identification and functional screens of uncharacterized proteins associated with eukaryotic ribosomal complexes. *Genes Dev.* 20, 1294–1307.
- 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.
- 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.
- Langdon, J., MacDonald, S.M., 2006. Assays for histamine-releasing factors: from identification and cloning to discovery of binding partners. *Methods Mol. Biol.* 315, 231–243.
- 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.
- Li, F., Zhang, D., Fujise, K., 2001. Characterization of fortilin, a novel antiapoptotic protein. *J. Biol. Chem.* 276, 47542–47549.
- 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.
- 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.
- Mayer, C., Appenzeller, U., Seelbach, H., Achatz, G., Oberkofler, H., Breitenbach, M., Blaser, K., Crameri, 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.
- Mohanty, A.K., Simmons, C.R., Wiener, M.C., 2003. Inhibition of tobacco etch virus protease activity by detergents. *Protein Expr. Purif.* 27, 109–114.
- 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.
- Rinnerthaler, M., Jarolim, S., Heeren, G., Palle, E., Perju, S., Klinger, H., Bogen-gruber, E., Madeo, F., Braun, R.J., Breitenbach-Koller, L., Breitenbach, M., Laun, P., 2006. MMI1 (YKL056c, TMA19), the yeast orthologue of the translationally controlled tumor protein (TCTP) has apoptotic functions and interacts with both microtubules and mitochondria. *Biochim. Biophys. Acta* 1757, 631–638.
- 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.
- Schleimer, R.P., MacGlashan Jr., D.W., Gillespie, E., Lichtenstein, L.M., 1982. Inhibition of basophil histamine release by anti-inflammatory steroids. II. Studies on the mechanism of action. *J. Immunol.* 129, 1632–1636.
- Schmid-Grendelmeier, P., Fluckiger, S., Disch, R., Trautmann, A., Wuthrich, B., Blaser, K., Scheynius, A., Crameri, 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., 1996. An immunoglobulin E-dependent recombinant histamine-releasing factor induces interleukin-4 secretion from human basophils. *J. Exp. Med.* 183, 1265–1270.
- Sheffield, P., Garrard, S., Derewenda, Z., 1999. Overcoming expression and purification problems of RhoGDI using a family of “parallel” expression vectors. *Protein Expr. Purif.* 15, 34–39.
- Shenton, D., Grant, C.M., 2003. Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Biochem. J.* 374, 513–519.
- Simon-Nobbe, B., Denk, U., Schneider, P.B., Radauer, C., Teige, M., Crameri, R., Hawranek, T., Lang, R., Richter, K., Schmid-Grendelmeier, P., Nobbe, S., Hartl, A., Breitenbach, M., 2006. NADP-dependent mannitol dehydrogenase, a major allergen of *Cladosporium herbarum*. *J. Biol. Chem.* 281, 16354–16360.
- Siraganian, R.P., 1975. Refinements in the automated fluorometric histamine analysis system. *J. Immunol. Methods* 7, 283–290.
- 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.
- Tuynder, M., Susini, L., Prieur, S., Besse, S., Fiucci, G., Amson, R., Telerman, A., 2002. Biological models and genes of tumor reversion: cellular reprogramming through tpt1/TCTP and SIAH-1. *Proc. Natl. Acad. Sci. U.S.A.* 99, 14976–14981.
- Velculescu, V.E., Zhang, L., Zhou, W., Vogelstein, J., Basrai, M.A., Bassett Jr., D.E., Hieter, P., Vogelstein, B., Kinzler, K.W., 1997. Characterization of the yeast transcriptome. *Cell* 88, 243–251.
- Venugopal, T., 2005. Evolution and expression of translationally controlled tumour protein (TCTP) of fish. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 142, 8–17.
- Vonakis, B.M., Gibbons Jr., S., Sora, R., Langdon, J.M., MacDonald, S.M., 2001. Src homology 2 domain-containing inositol 5' phosphatase is negatively associated with histamine release to human recombinant histamine-releasing factor in human basophils. *J. Allergy Clin. Immunol.* 108, 822–831.
- Wantke, F., MacGlashan, D.W., Langdon, J.M., MacDonald, S.M., 1999. The human recombinant histamine releasing factor: functional evidence that it does not bind to the IgE molecule. *J. Allergy Clin. Immunol.* 103, 642–648.

- Yan, L., Fei, K., Bridge, D., Sarra 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.
- Zhang, D., Li, F., Weidner, D., Mnjoyan, Z.H., Fujise, K., 2002. Physical and functional interaction between myeloid cell leukemia 1 protein (MCL1) and Fortilin. The potential role of MCL1 as a fortilin chaperone. *J. Biol. Chem.* 277, 37430–37438.
- Zhao, Z.S., Granucci, F., Yeh, L., Schaffer, P.A., Cantor, H., 1998. Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. *Science* 279, 1344–1347.
- 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.