

Characterization of a Novel Isoform of α -Nascent Polypeptide-associated Complex as IgE-defined Autoantigen

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The nascent polypeptide-associated complex is required for intracellular translocation of newly synthesized polypeptides in eukaryotic cells. It may also act as a transcriptional coactivator in humans and various eukaryotic organisms and binds to nucleic acids. Recently, we provided evidence that a component of nascent polypeptide-associated complex, α -nascent polypeptide-associated complex, represents an IgE-reactive autoantigen for atopic dermatitis patients. By oligonucleotide screening we isolated a complete cDNA coding for a so far unknown α -nascent polypeptide-associated complex isoform from a human epithelial cDNA library. Southern blot hybridization experiments provided further evidence that α -nascent polypeptide-associated complex is encoded by a gene family. Recombinant α -nascent polypeptide-associated complex was expressed in *Escherichia coli* as a soluble, His-tagged protein, and purified via nickel affinity chromatography. By circular dichroism analysis it is demonstrated that purified recombinant α -nascent polypeptide-associated complex represents a folded

protein of mixed α -helical and β -sheet conformation with unusual high thermal stability and remarkable refolding capacity. Complete recombinant α -nascent polypeptide-associated complex (215 amino acids) and its 86 amino acid C-terminal fragment specifically bound IgE autoantibodies. Recombinant α -nascent polypeptide-associated complex also inhibited IgE binding to natural α -nascent polypeptide-associated complex, demonstrating the presence of common IgE epitopes between the recombinant and natural protein. Furthermore, recombinant α -nascent polypeptide-associated complex induced specific lymphoproliferative responses in peripheral blood mononuclear cells of a sensitized atopic dermatitis patient. As has been proposed for environmental allergens it is possible that T cell responses to IgE-defined autoantigens may contribute to the chronic skin manifestations in atopic dermatitis. **Key words:** allergy/atopic dermatitis/autoantigen/circular dichroism/nascent polypeptide-associated complex. *J Invest Dermatol* 119:820–829, 2002

Atopy is a genetically determined hypersensitivity disease that is based on the production of IgE antibodies against *per se* harmless antigens (i.e., allergens) (Kay, 1997). Allergens can be derived from a variety of environmental allergen sources (e.g., pollens, mites, moulds, animals, food) and may induce a variety of allergic manifestations, including allergic rhinoconjunctivitis, allergic asthma, food allergy, anaphylaxis, and atopic dermatitis (AD). AD is different from other forms of acute allergic manifestations as it mainly resembles features of a cell-mediated, type IV-like immune reaction (Werfel *et al*, 1996; Herz *et al*, 1998; Wistokat-Wulfig *et al*, 1999; Leung, 2000; Wollenberg and Bieber, 2000). In addition, AD patients frequently contain high

levels of IgE antibodies directed against a variety of exogenous allergens. Recently, we found that a large proportion of patients suffering from AD exhibit IgE reactivity also to human proteins, which are expressed in a variety of cell and tissue types (Valenta *et al*, 1996).

Using serum IgE autoantibodies from a patient suffering from severe AD we have isolated cDNAs coding for IgE-reactive autoantigens (Valenta *et al*, 1998). One of these cDNA coded for a C-terminal portion of α -nascent polypeptide-associated complex (α -NAC) (Natter *et al*, 1998), a phylogenetically well conserved protein (humans, animals, insects, plants, yeast), which represents a component of the NAC (Wiedmann *et al*, 1994). It is likely one of the first cytosolic factors that contacts nascent polypeptides when they emerge from the ribosome and is involved in the correct targeting and translocation of intracellular polypeptides (Wang *et al*, 1995). In addition, α -NAC was described as developmentally regulated transcriptional coactivator (Yotov and Arnaud, 1996) and recently was shown to bind to nucleic acids (Beatrix *et al*, 2000; Whitby and Dixon, 2001).

Here we report the molecular characterization of the IgE autoantigen, α -NAC, which according to the allergen nomenclature

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Abbreviations: α -NAC, α -nascent polypeptide-associated complex; AD, atopic dermatitis;

Hom s 2.01	ATG	CCG	GGC	GAA	GCC	ACA	GAA	ACC	GTC	CCT	GCT	ACA	GAG	CAG	42
α NAC	---	---	C---	---	---	---	---	---	---	---	---	---	---	---	
Hom s 2.01	GAG	TTG	CCG	CAG	TCC	CAG	GCT	GAG	ACA	GGG	TCT	GGA	ACA	GCA	84
α NAC	---	---	---	---	C---	---	---	---	---	---	---	---	---	-A-	
Hom s 2.01	TCT	GAT	AGT	GGT	GAA	TCA	GTA	CCA	GGG	ATT	GAA	GAA	CAG	GAT	126
α NAC	---	---	C---	---	-A-	---	---	---	-A-	C---	---	---	---	---	
Hom s 2.01	TCC	ACC	CAG	ACC	ACC	ACA	CAA	AAA	GCC	TGG	CTG	GTG	GCA	GCA	168
α NAC	---	---	---	G-A	---	---	---	C---	---	CA-	---	-C-	---	---	
Hom s 2.01	GCT	GAA	ATT	GAT	GAA	GAA	CCA	GTC	GGT	AAA	GCA	AAA	CAG	AGT	210
α NAC	---	---	---	---	---	---	---	---	A-	---	---	---	---	---	
Hom s 2.01	CGG	AGT	GAA	AAG	AGG	GCA	CGG	AAG	GCT	ATG	TCC	AAA	CTG	GGT	252
α NAC	---	---	---	---	-A-	---	---	---	---	---	---	---	---	---	
Hom s 2.02															
Hom s 2.01	CTT	CTA	CAG	GTT	ACA	GGA	GTT	ACT	AGA	GTC	ACT	ATC	TGG	AAA	294
α NAC	---	-GG	---	---	---	---	---	---	---	---	---	---	C-	---	
Hom s 2.02	---	-GG	---	---	---	---	---	---	---	---	---	---	C-	---	
Hom s 2.01	TCT	AAG	AAT	ATC	CTC	TTT	GTC	ATC	ACA	AAA	CTG	GAC	GTC	TAC	336
α NAC	---	---	---	-A	---	---	---	---	---	---	-CA	-T	---	---	
Hom s 2.02	---	---	---	---	---	---	---	---	---	---	-CA	-T	---	---	
Hom s 2.01	AAG	AGC	CCT	GCT	TCG	GAT	GCC	TAC	ATA	GTT	TTT	GGG	GAA	GCC	378
α NAC	---	---	---	---	-A	---	A-T	---	---	---	---	---	---	---	
Hom s 2.02	---	---	---	---	-A	---	A-T	---	---	---	---	---	---	---	
Hom s 2.01	AAG	ATC	CAA	GAT	TTA	TCT	CAG	CAA	GCA	CAA	CTA	GCA	GCT	GCG	420
α NAC	---	---	G-	---	---	-C	---	---	---	---	---	---	---	-T	
Hom s 2.02	---	---	G-	---	---	-C	---	---	---	---	---	---	---	-T	
Hom s 2.01	GAG	AAA	TTC	AGA	GTT	CAA	GGT	GAA	GCT	GTC	GGA	AAC	ATT	CAA	462
α NAC	---	---	---	-A-	---	---	---	---	---	---	TC-	---	---	---	
Hom s 2.02	---	---	---	-A-	---	---	---	---	---	---	TC-	---	---	---	
Hom s 2.01	GAA	AAC	ACA	CAG	ACT	CCA	ACT	GTA	CAA	GAG	GAG	AGT	GAA	GAG	504
α NAC	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
Hom s 2.02	---	---	---	---	---	---	---	---	---	---	---	-C	---	---	
Hom s 2.01	GAA	GAG	GTC	GAT	GAA	ACA	GGT	GTA	GAA	GTT	AAA	GAC	GTG	AAA	546
α NAC	---	---	---	---	---	---	---	---	---	---	-G	---	A-T	G-T	
Hom s 2.01	TTG	GTC	ATG	TCA	CAA	GCA	AAT	GTG	TCG	AGA	GCA	AAG	GCA	GTC	588
α NAC	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
Hom s 2.01	CGA	GCT	CTG	AAG	AAC	AAC	AGT	AAT	GAT	ATT	GTA	AAT	GCG	ATT	630
α NAC	---	-C	---	---	---	---	---	---	---	---	---	---	---	---	
Hom s 2.01	ATG	GAA	TTA	ACA	GTG	TAA	cca	tct	gaa	agc	aac	ttt	ttt	tgg	672
α NAC	---	---	---	---	A-	---	---	-a-	-g-	---	---	---	---	---	
Hom s 2.01	tgt	ctc	aaa	gga	gta	act	gca	gct	tgg	ttt	gaa	att	tgt	act	714
α NAC	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
Hom s 2.01	gtt	tct	atc	ata	aat	aaa	gtt	atg	act	tct	tgc	tgg	aaa	aag	ccc gaa ttc 765
α NAC	---	---	---	---	---	---	---	---	g-	---	-t	---	---	---	aaa aaa aaa

Figure 1. Comparison of the cDNA sequence of Hom s 2.01 with the cDNA sequence of human α -NAC and a partial Hom s 2.02 cDNA. Nucleotides of the coding region were printed in capital letters, whereas those of the noncoding regions are shown in lower case letters. Identical nucleotides were indicated by dashes in the alignment. *Eco*RI sites are printed in italics and are underlined. The stop codon is indicated by an asterisk. The DNA and deduced amino acid sequence of Hom s 2.01 have been submitted to the EMBL Nucleotide Sequence Database under the accession number: AJ278883.

ure system was designated Hom s 2 (Natter *et al*, 1998). We used an oligonucleotide specific for the 3' end of the α -NAC cDNA to screen a cDNA library prepared from the human epithelial cell line, A431 for α -NAC/Hom s 2 isoforms.

The possibility that several α -NAC isogenes may exist in humans was analyzed by southern blot hybridization experiments. A complete α -NAC/Hom s 2 cDNA as well as the C-terminal portion of α -NAC/Hom s 2 were expressed in *Escherichia coli* to demonstrate the IgE binding capacity of the recombinant IgE autoantigen and to determine its IgE-reactive domains. Structural fold of recombinant Hom (rHom) s 2 was analyzed by circular dichroism spectroscopy. Furthermore, we investigated whether rHom s 2 can induce specific lymphoproliferative responses in a sensitized AD patient.

MATERIALS AND METHODS

Phage libraries, *E. coli* strains, and plasmids A cDNA library constructed from the human epithelial cell line A431 in phage λ gt11 was purchased from Clontech (Palo Alto, CA). *E. coli* strains Y1090 [*hsd* ($r_k^- m_k^+$) *lac* U169, *ProA*⁺, *Ion*⁻, *araD* 139, *StrA*, *Sup* *Ftrp*C22:Tn10(pmC9)] and Y1089 [*hsd* ($r_k^- m_k^+$) *lac* U169, *ProA*⁺, *Ion*⁻, *araD* 139, *StrA*, *hflA* 150 chr:Tn10(pmC9)] were obtained from Amersham (Amersham, Buckinghamshire, U.K.). *E. coli* strain XL-1 Blue: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F'*proAB lacIqZΔEM15* Tn10(Tetr)]_c was from Stratagene (La Jolla, CA) and *E. coli* strain BL21(DE3): F'*ompT r_B^- m_B^-*(DE3) was from Novagen (Cambridge, U.K.). Plasmid pUC19 used for subcloning was from Boehringer (Mannheim, Germany) and plasmid pET-17b used for overexpression in *E. coli* was from Novagen.

Sera, cell lines, and tissue specimens Sera were obtained from AD patients diagnosed according to Hanifin and Rajka (1980), from patients with allergic rhinoconjunctivitis and from nonatopic individuals by venipuncture. The diagnosis of type I allergy was based on case history, demonstration of specific IgE against environmental allergen sources by RAST measurements and positive skin prick reactivity as described (Niederberger *et al*, 1998). All sera were stored at -20°C until use. A human epithelial cell line (A431) derived from an epidermoid mamma carcinoma was obtained from American Type Culture Collection (Rockville, MD) and grown as described (Valenta *et al*, 1996). Human tissue specimens containing natural Hom s 2 were obtained from biopsies taken either *ex vivo* (colon) or immediately post mortem (brain-medulla) for routine histology.

IgE immunoblotting Protein extracts were prepared by homogenizing tissues in sodium dodecyl sulfate (SDS) sample buffer (Laemmli, 1970), and boiling of the extracts for 5 min. Insoluble material was removed by centrifugation in a bench fuge (12,000 r.p.m., 5 min, room temperature). Protein extracts were separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fling and Gregerson, 1986) and stained with Coomassie blue (Bradford, 1976). Proteins were transferred on to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by electroblotting (Towbin *et al*, 1979), and membrane-bound proteins were exposed to serum IgE as described (Valenta *et al*, 1996). Bound IgE antibodies were detected with ¹²⁵I-labeled anti-human IgE antibodies (RAST, Pharmacia & Upjohn, Uppsala, Sweden) and visualized by autoradiography. A monoclonal anti-Histag antibody was obtained from Novagen.

Oligonucleotide screening of the A431 cDNA library In order to obtain a complete Hom s 2 cDNA clone, we designed an oligonucleotide probe according to the incomplete Hom s 2.02 cDNA isolated by IgE immunoscreening (Natter *et al*, 1998). The oligonucleotide 5'-GAC TCT AGT AAC TCC TGT AAC CTG CCG AAG ACC C-3' was ³²P-labeled using ³²P γ -adenosine triphosphate and polynucleotide kinase (Boehringer). Approximately 250,000 pfu (plaque-forming units) of the A431 cDNA library were used to infect *E. coli* Y1090 and plated at a density of 20,000 phage per plate (140 mm diameter) and nitrocellulose filter replicas (Schleicher & Schuell) were hybridized with the ³²P-labeled Hom s 2.02-specific oligonucleotide.

Isolation and molecular characterization of a Hom s 2.01-encoding cDNA clone Two positive clones were obtained after the first round of screening and recloned to homogeneity by three further rounds of oligonucleotide screening. Phage DNA was isolated from the positive clones and the cDNA inserts were excised with *EcoRI*, gel purified, and

ligated into the *EcoRI* site of plasmid pUC 19 (Boehringer). Plasmids were transformed into *E. coli* XL1-Blue and plasmid DNA was isolated using Qiagen tips (Qiagen, Hilden, Germany). Both cDNA strands were sequenced using forward and reversed primers (Boehringer), internal primers designed according to the Hom s 2 cDNA sequence (MWG-Biotech, Ebersberg, Germany), ³⁵S-deoxycytidine triphosphate (NEN, Stevenage, U.K.) and a T7 sequencing kit (Pharmacia, Uppsala, Sweden) (Sanger *et al*, 1977). All molecular biologic manipulations were performed as described in (Sambrook *et al*, 1989).

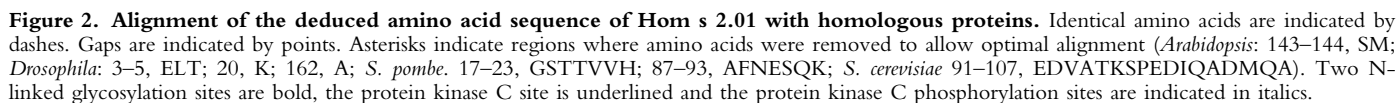
The McVector program (Kodak Rochester, NY) was used to establish the amino acid sequence of the Hom s 2.01 open reading frame, to calculate pI, molecular weight, amino acid composition, secondary structure of Hom s 2, and to search for sequence motifs. The cDNA and deduced amino acid sequence of Hom s 2.01 were compared with the sequences deposited in GenBank using the BLAST program.

Southern blot experiments A431 genomic DNA was isolated by cesium chloride density centrifugation (Sambrook *et al*, 1989). Five microgram aliquots of genomic DNA were digested with either *HindIII*, *BamHI*, *KpnI*, *PstI*, or *EcoRI*, separated by 1% agarose gel electrophoresis, blotted on to nitrocellulose, and hybridized with the complete ³²P-deoxycytidine triphosphate-labeled Hom s 2.01 cDNA (Feinberg and Vogelstein, 1983). Five micrograms of *PstI*-digested λ phage DNA was run on the same gel as a molecular weight standard. The southern blot was washed up to a final stringency of 0.75 $\times$ sodium citrate/chloride buffer, 0.1% SDS, 65°C, and was exposed to KODAK X-OMAT films using intensifying screens at -70°C.

Expression of complete rHom s 2.01 in *E. coli* The coding region of the Hom s 2.01 cDNA was polymerase chain reaction amplified using the following primer pair: Hom s 2.01 forward: 5'-GGG ATT CCA TAT GCC GGG CGA AGC CAC AGA AAC C-3'; Hom s 2.01 reversed: 5'-GGG ATT CCA TAT GTT AGT GGT GGT GGT GGT GGT GCA CTG MA ATT OCA TAA TCG CAT-3'. The forward and reversed primer contained *NdeI* (underlined) sites to allow subcloning into the *NdeI* site of plasmid pET-17b (Novagen). The thus inserted Hom s 2.01 cDNA should allow the production of authentic rHom s 2.01 containing a C-terminal hexahistidine tag. *E. coli* BL21(DE3) (Novagen) were transformed with plasmid pET-17b containing the Hom s 2.01 cDNA and positive clones were isolated by colony screening using serum from an AD patient containing Hom s 2-specific IgE antibodies and an anti-histag antibody (Novagen). For the expression in liquid culture, *E. coli* BL21 (DE3) were transformed with plasmid pET 17b-Hom s 2.01 or, for control purposes, with plasmid pET-17b alone. Fifty milliliter aliquots of Luria Broth containing 100 μ g per ml ampicillin were inoculated with one colony obtained after transformation, grown to an OD₆₀₀ of 0.3 and induced by addition of IPTG (isopropyl- β -D-thiohalacopsranoside) to a final concentration of 1 mM for 4 h. *E. coli* cells were harvested by centrifugation and equal aliquots of the harvested cells were lysed in SDS sample buffer and analyzed by SDS-PAGE and Coomassie blue staining to visualize overexpression of rHom s 2.01. The rest of the cellular pellet was stored at -20°C until use.

Expression of the C-terminal Hom s 2.02 fragment in *E. coli* An 86 amino acid C-terminal fragment of Hom s 2 (Hom 2.02: Fig 1) was expressed as β -galactosidase (β -gal) fusion protein in *E. coli* Y1089. *E. coli* Y1089 were infected with λ gt11 phage containing the Hom 2.02 cDNA (Fig 1) or, for control purposes, with empty λ gt11 phage. Lysogenic *E. coli* Y1089 were grown in Luria Broth medium containing 100 mg per liter ampicillin, 0.4% w/v maltose, and 10 mM MgCl₂ at 32°C until an OD₆₀₀ of 0.5. The culture temperature was then rapidly shifted to 42°C for 30 min, and expression of the β -gal-fused Hom s 2.02 fragment or β -gal alone was induced at 37°C by addition of IPTG to a final concentration of 5 mM. Bacterial cells were collected by centrifugation and stored at -20°C until use.

Purification of His-tagged rHom s 2.01 via nickel affinity chromatography *E. coli* BL21(DE3) containing the Hom s 2-expressing pET-17b construct were grown in Luria Broth containing ampicillin to an OD₆₀₀ of 0.3. Protein expression was induced by addition of IPTG to a final concentration of 0.5 mM and growth at 32°C. Cells were harvested by centrifugation and homogenized in 25 mM imidazole pH 8.0, 0.1% Triton, 1 mM phenylmethylsulfonyl fluoride using an ultraturax (Ika, Staufen, Germany). Insoluble material was removed by centrifugation at 30,000 $\times$ g at 4°C and the supernatant was dialyzed against 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, and 10 mM imidazole. The dialysate was applied to Ni-NTA sepharose (Qiagen). The Ni-NTA sepharose column was then washed with two volumes of 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 20 mM



IgE reactivity of rHom s 2.01 and the rHom s 2.02 fragment Comparable amounts of *E. coli* Y1089 expressing β -gal fused Hom s 2.02 or β -gal alone were extracted in SDS sample buffer, separated by SDS-PAGE (12.5% SDS-PAGE: rHom s 2.01; 8% SDS-PAGE: β -gal fused Hom s 2.02), and blotted on to nitrocellulose. Nitrocelluloses were incubated with serum from an AD patient with and without Hom s 2-specific IgE but containing comparable levels of total

IgE reactivity to purified rHom s 2 was studied by Western blot and enzyme-linked immunosorbent assay. Nitrocellulose membranes containing purified Hom s 2 (5 µg per cm preparative gel) were incubated with sera from atopic patients with or without Hom s 2-specific IgE and with serum from a nonatopic individual as described (Natter *et al.*, 1998). Bound IgE antibodies were detected with ¹²⁵I-labeled anti-human IgE antibodies (Pharmacia) and visualized by autoradiography. For enzyme-

linked immunosorbent assay detection of Hom s 2-specific IgE and IgG subclass responses, enzyme-linked immunosorbent assay plates (Nunc Maxisorb, Roskilde, Denmark) were coated with 5 µg per ml of purified rHom s 2 and for control purposes, with the purified dog allergen, Can f 3 (Spitzauer *et al*, 1995). Specific IgE and IgG1–4 subclass responses were detected as described (Vrtala *et al*, 1996). Results are displayed as mean values with an experimental error of <5%.

IgE inhibition experiments Cross-reactivity of rHom s 2 with its human colon-derived natural counterpart was investigated by IgE immunoblot inhibition experiments. Bacterial extracts containing rHom s 2.01 or without rHom s 2.02 were obtained by homogenization of *E. coli* BL21 (DE3) in phosphate-buffered saline with an ultraturrax. Sera from a Hom s 2 reactive and another AD patient without Hom s 2-specific IgE or serum dilution buffer (buffer A: 50 mM Na phosphate pH 7.5, 0.5% w/v bovine serum albumin, 0.5% v/v Tween 20, 0.05% w/v NaN₃) were preadsorbed with equal amounts of bacterial protein extracts (1 ml of a 1 : 10 diluted serum was preadsorbed with 100 µl containing approximately 100 µg bacterial protein extract) overnight at 4°C. Nitrocellulose strips containing blotted human tissue extracts were blocked in buffer A and incubated with the pretreated sera or buffer overnight at 4°C. Bound IgE antibodies were detected with ¹²⁵I-labeled anti-human IgE antibodies (RAST, Pharmacia) as described (Natter *et al*, 1998).

Circular dichroism analysis Circular dichroism measurements were performed in MilliQ water pH 7.3 with a protein concentration of 1.12×10^{-5} M as determined by BCA assay (BCA Protein assay Reagent, Pierce). The investigations were carried out on a Jasco J-715 spectropolarimeter using a 0.1 cm pathlength cell with cooling jacket connected to a water thermostating device. The sample was heated to 95°C with a heat rate of 1°C per min and cooled down using the same parameters. A continuous temperature scan was performed at 222 nm with a step resolution of 0.5°C and a wait time of 1 min. Spectra were recorded at 20, 40, 60, 80, and 95°C with 0.2 nm resolution at a scan speed of 50 nm per min and resulted from averaging five scans. The final spectra were baseline corrected by subtracting the corresponding MilliQ spectra obtained under identical conditions. Results were expressed as the mean residue ellipticity [Θ] at a given wavelength. The data were fitted with the secondary structure estimation program J-700 (J-700 for Windows Secondary Structure Estimation, version 1.10.00, copyright 1993–94 JASCO Corp., Tokyo, Japan) according to (Yang *et al*, 1986) using the finite-mode fitting procedure.

Lymphoproliferation assays Peripheral blood mononuclear cells (PBMC) were isolated from AD patients by Ficoll (Amersham Pharmacia Biotech, Little Chalfont, U.K.) density gradient centrifugation. AD patients containing comparable levels of total IgE with and, for control purposes, without Hom s 2-specific IgE antibodies were investigated. PBMC (2×10^5) were cultured in triplicates in 96-well plates (Nunc, Nalgen Nunc International, Roskilde, Denmark) in 200 µl serum-free Ultra Culture medium (BioWhittaker, Rockland, ME) supplemented with 2 mM L-glutamine (Sigma, St Louis, MO), 50 µM β-mercaptoethanol (Sigma) and 0.1 mg gentamicin per ml (Sigma) at 37°C using 5% CO₂ in a humidified atmosphere. Cells were stimulated with different concentrations (0.6–5 µg per well) of Hom s 2 and, for control purposes, with an environmental allergen, the dog allergen Can f 3 (dog albumin), 4 U interleukin-2 per well (Boehringer) or medium alone. After 6 d culture, 0.5 µCi per well ³H-thymidine (Amersham Pharmacia Biotech) was added and 16 h thereafter incorporated radioactivity was measured by liquid scintillation counting using a microbeta scintillation counter (Wallac ADL, Freiburg, Germany) and mean cpm were calculated from the triplicates. The stimulation index (SI) was calculated as the quotient of the cpm obtained by antigen or interleukin-2 stimulation and the unstimulated medium control. All proliferation experiments were repeated at least once.

RESULTS

Isolation and characterization of a cDNA coding for a complete α-NAC/Hom s 2.01 isoform The cDNA isolated by screening with an oligonucleotide specific for the partial Hom s 2 cDNA, represented an isoform with nucleotide exchanges in 31 triplets compared with the α-NAC cDNA sequence (Fig 1). An open reading frame of 645 base pairs, including the methionine-encoding start codon (ATG) coded for a Hom s 2.01 polypeptide of 215 amino acids. The partial cDNA clone isolated by IgE

immunoscreening out of the same cDNA library coded for a 86 amino acid long fragment.

C-terminal Hom s 2 portion (Natter *et al*, 1998), which was identical to the previously published α-NAC sequence, however, was different from the Hom s 2.01 sequence and therefore was designated according to the Allergen nomenclature Hom s 2.02. The Hom s 2.01 protein deduced from the cDNA sequence represents an acidic polypeptide (calculated pI: 4.48) with a calculated molecular mass of 23.2 kDa. It is rich in alanine (11.16%), valine (10.23%), and glutamic acid (11.63%) residues and lacks cysteines and histidines. Amino acid residues with high surface probability, flexibility, and high antigenic index were predicted to be scattered throughout the protein. The secondary structure prediction according to Chou and Fasman (1974) and Garnier *et al* (1978) indicated that the protein consists mostly of α-helical secondary structure with only one area assuming predicted β-sheet conformation (amino acids 80–120). A search for protein motifs revealed the presence of two N-linked glycosylation sites (Fig 2: bold), one protein kinase C site (Fig 2: underlined), and two protein kinase C phosphorylation sites (Fig 2: italics).

Hom s 2 is homologous to α-NAC-related proteins from various species When the databases for proteins with significant sequence homology to Hom s 2.01 were searched, several highly homologous proteins were found in mouse, fruit fly, plants (*Arabidopsis thaliana*), yeast, and protozoa (Fig 2). Hom s 2.01 can be considered a true isoform of human α-NAC because it contained 21 amino acid exchanges compared with human α-NAC.

The deduced amino acid sequence of Hom s 2.01 shared the highest degree of end to end sequence identity with the α-NAC protein from mouse (90.23%). Significant homologies were found with putative α-NAC sequences found in *Arabidopsis* (45.12%) and fruit fly (*Drosophila melanogaster*) (52.55%). The Hom s 2.02 homologs from *Schizosaccharomyces pombe* and *S. cerevisiae* were shorter than the other α-NAC proteins and several amino acids had to be removed to allow optimal sequence alignment. The sequence identity between Hom s 2.01 and homologs from *S. pombe* and *S. cerevisiae* were 42.77% and 36.13%, respectively. Hom s 2.01 homologous sequences were found also in protozoa (*Leishmania donovani*), which exhibited a 39.55% sequence identity with Hom s 2.01. The N-linked glycosylation sites, phosphorylation sites and protein kinase C sites predicted for Hom s 2.01 were conserved in α-NAC proteins of mammalian origin. Overall, we noticed that the C-terminal portions of the Hom s 2.01-related proteins that contained a major IgE-reactive domain represented the most conserved protein domains among the α-NAC homologous proteins.

The Hom s 2.01 cDNA hybridizes with several restriction fragments in human genomic DNA: evidence for a Hom s 2 gene family Our finding that the Hom s 2.01 cDNA differed from the α-NAC cDNA sequence indicated the occurrence of several α-NAC isogenes (Figs 1 and 2). Hybridization of human genomic DNA digested with enzymes that do not cut in the Hom s 2.01 cDNA (Fig 3: *Hind*III, *Bam*HI, *Kpn*I, *Pst*I, *Eco*RI) showed that several restriction fragments hybridized with the Hom s 2.01 cDNA: At least four hybridizing fragments were detected in *Pst*I- and *Eco*RI-digested DNA, three fragments in *Hind*III-digested DNA bound to the probe and two fragments in *Bam*HI- and *Kpn*I-digested DNA were detected by the Hom s 2.01 cDNA. All but one restriction fragment (a 600 base pair fragment in *Pst*I-digested DNA) were larger than the Hom s 2.02 cDNA. Although we have at present no information regarding the occurrence of introns in the Hom s 2/α-NAC gene, the hybridization data together with the Hom s 2.01 sequence strongly indicate that human α-NAC is encoded by a gene family.

Identification of the C-terminal portion of Hom s 2 as an IgE-reactive fragment In order to determine whether the C-terminal portion of Hom s 2 contains IgE epitopes, we tested the

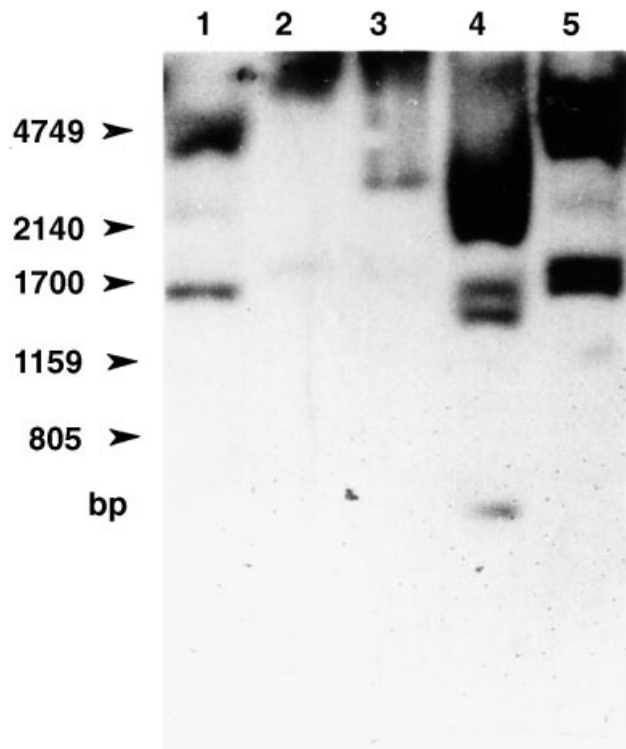


Figure 3. Hybridization of the Hom s 2.01 cDNA with nitrocellulose blotted human genomic DNA prepared from A431 cells. Five microgram aliquots of genomic DNA were digested with *Hind*III (lane 1), *Bam*HI (lane 2), *Kpn*I (lane 3), *Pst*I (lane 4), and *Eco*RI (lane 5), separated by 1% agarose gel electrophoresis and blotted on to nitrocellulose. The membrane was hybridized with the complete Hom s 2.01 cDNA (Fig 1). Molecular weights (*Pst*I digested λ DNA) are displayed on the left margin in base pairs (bp).

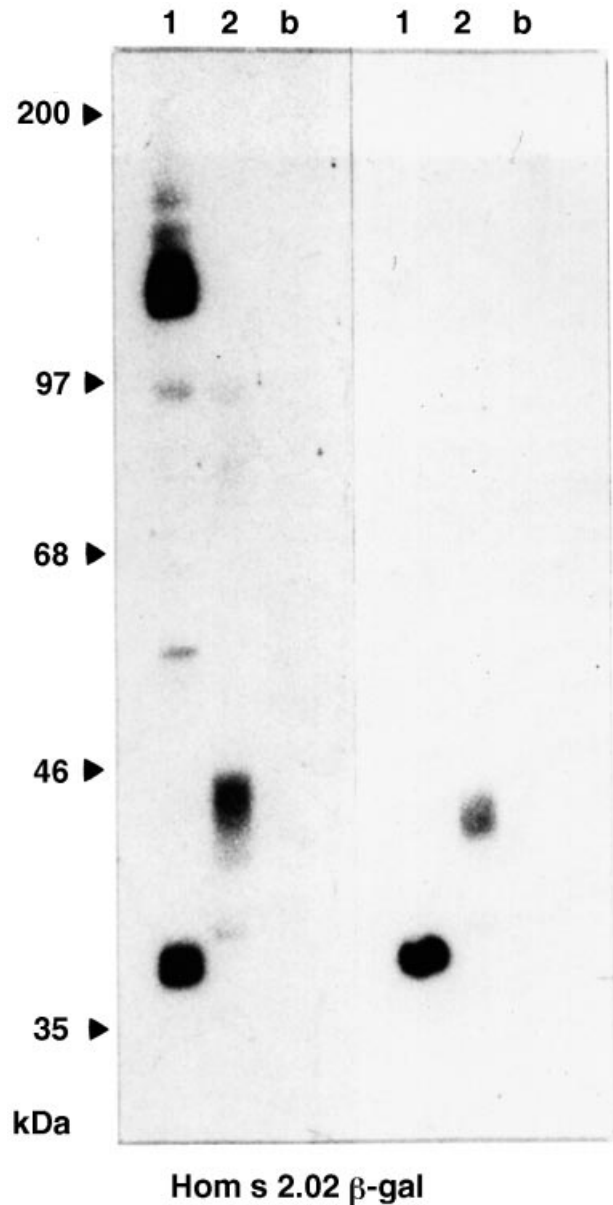


Figure 4. IgE binding capacity of a recombinant C-terminal Hom s 2.02 fragment. The C-terminal Hom s 2.02 fragment was expressed as a β -gal fusion protein. Nitrocellulose-blotted bacterial extracts containing the β -gal-fused C-terminal Hom s 2.02 fragment (block: Hom s 2.02) or β -gal alone (block: β -gal) were exposed to serum IgE from an AD patient with (lane 1) and without (lane 2) Hom s 2-specific IgE. Both sera contained comparable levels of total serum IgE. Lane b represents the buffer controls. Bound IgE antibodies were detected with ^{125} I-labeled anti-human IgE antibodies and visualized by autoradiography. Molecular weights (kDa) are indicated on the left margin.

recombinant C-terminal β -gal-fused Hom s 2 (Hom s 2.02) fragment and, for control purposes, β -gal alone with serum IgE from a Hom s 2-reactive AD patient. Serum IgE of the Hom s 2-reactive patient (Fig 4: lane 1) but not from an AD patient containing comparable levels of total IgE (Fig 4: lane 2) reacted with the β -gal-fused C-terminal portion of Hom s 2 at approximately 120 kDa. The molecular weight of the IgE-reactive band is due to the fusion of the C-terminal Hom s 2 fragment with the 110 kDa β -gal. When extracts were incubated with buffer but without addition of serum (Fig 4: lane b) no reactivity was observed.

Purification of complete rHom s 2.01 via nickel affinity chromatography The SDS-PAGE analysis of extracts from induced *E. coli* BL21 (DE3) containing the Hom s 2.01-expressing plasmid revealed overexpression of rHom s 2.01 at approximately 33 kDa and of a protein band of >200 kDa (Fig 5: Extract—Coomassie, lane +). Using nickel affinity chromatography, a rHom s 2.01 could be purified as a major component of 33 kDa (Fig 5: Coomassie, purified). Typical expression yields were in the range of 3–5 mg protein per liter culture. In addition to the 33 kDa component a moiety of >200 kDa and a few weak bands of <33 kDa were copurified by nickel affinity chromatography, which reacted with the monoclonal anti-Histag antibody and with serum IgE from a Hom s 2-reactive AD patient (Fig 5: IgE, lane 4). AD patients containing comparable levels of total serum IgE as the Hom s 2-reactive patient did not show IgE reactivity with purified rHom s 2.01 (Fig 5: lanes 1–3). Sera from a rhinoconjunctivitis patient and a nonatopic individual without Hom s 2-specific IgE

did not exhibit IgE reactivity with the affinity purified rHom s 2.01 (Fig 5: lanes 5 and 6).

Recombinant *E. coli*-expressed Hom s 2.01 inhibits IgE binding to its natural counterpart Next we were interested to study whether *E. coli*-expressed Hom s 2.01 shares IgE epitopes with its natural counterpart. As tissues of the gastrointestinal tract are rich in α -NAC (Wiedmann *et al*, 1994) we prepared extracts from human colon tissue specimens. Preincubation of serum from a Hom s 2-reactive AD patient with rHom s 2.01 (Fig 6: lane 1a) but not with bacterial extract alone (Fig 6: lane 1) lead to a reduction of IgE reactivity to 30 kDa, 14 kDa, and 8 kDa components in

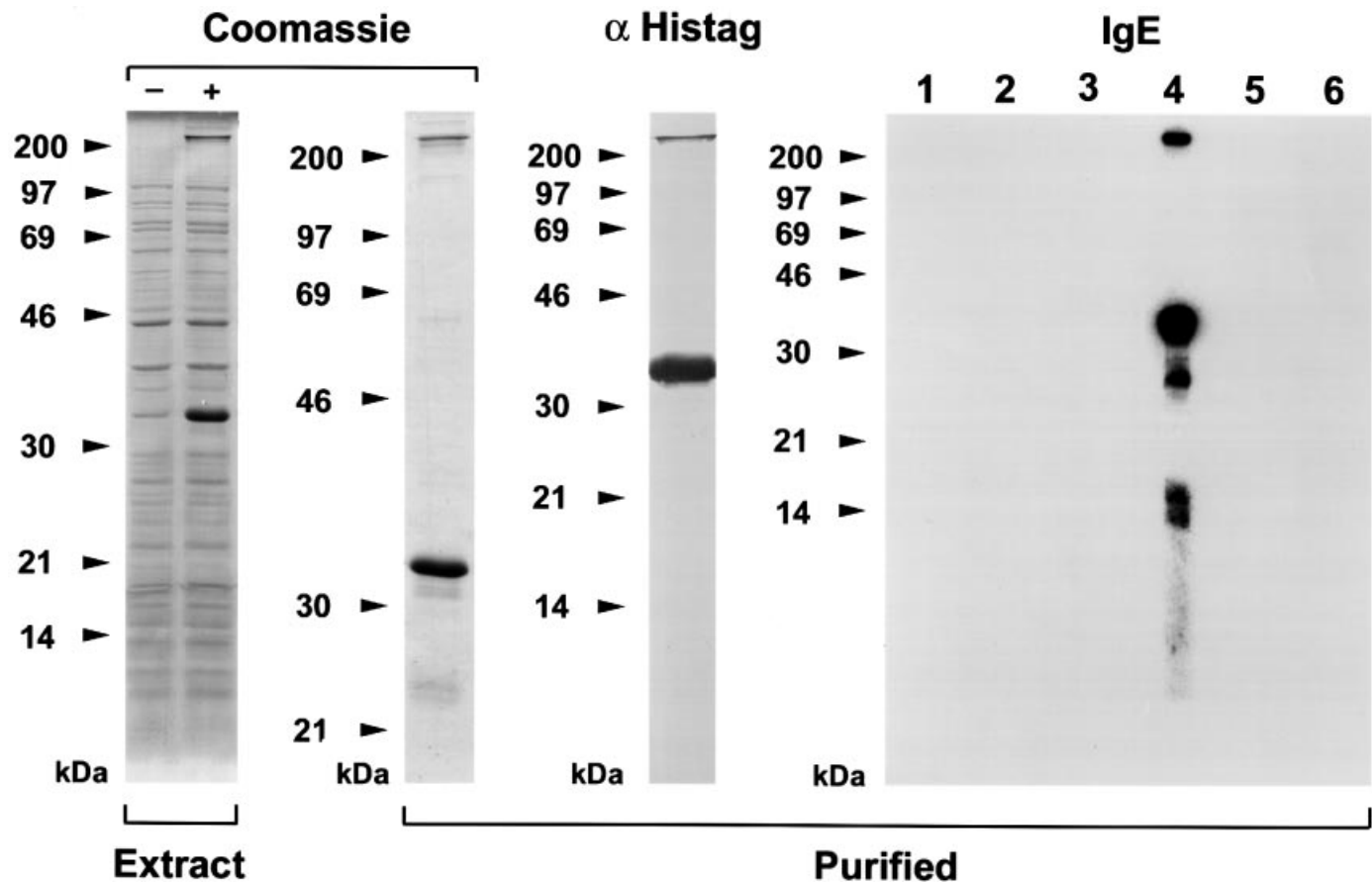


Figure 5. Expression of rHom s 2.01 in *E. coli*. Total protein extracts from *E. coli* BL21 (DE3) obtained before (–) and after induction (+) of protein expression with IPTG and purified rHom s 2.01 were separated by SDS–PAGE and stained with Coomassie blue. Nitrocellulose-blotted purified rHom s 2.01 was exposed to an alkaline phosphatase conjugated anti-Histag antibody (α Histag), to serum IgE from four patients with AD (lanes 1–4), a patient with allergic rhinoconjunctivitis (lane 5) and a nonatopic individual (lane 6). Molecular weights are displayed in kDa on the right margins.

blotted colon extracts. Serum from an AD patient without Hom s 2-specific IgE did not exhibit IgE reactivity to nitrocellulose-blotted human colon extract (**Fig 6**: lanes 2 and 2a). When blots were incubated with buffer without addition of serum no reactivity was observed (**Fig 6**: lanes 3 and 3a). Similar results were obtained when IgE inhibition studies were performed with nitrocellulose-blotted extracts from human stomach and brain: rHom s 2 inhibited IgE binding of the Hom s 2-reactive serum to components of approximately 30 kDa and lower molecular weights (data not shown).

rHom s 2 is a thermostable protein of mixed α -helical and β -sheet conformation with high refolding capacity The far ultraviolet spectra indicate that the protein contains a considerable amount of secondary structure. The spectra are dominated by an α -helical minimum at 208 nm, a characteristic rise towards a maximum below 195 nm and a shoulder between 215 and 220 nm (**Fig 7**). Fitting the secondary structure yields a mixed α/β -fold with 18.5% α -helix content (data not shown). This corresponds with the results derived from secondary structure prediction based on the amino acid sequence (PHD program: Rost and Sander, 1993; Rost, 1996) (data not shown). The rHom s 2.01 protein turns out to be remarkably stable in heat denaturation experiments. In a temperature scan a reversible folding transition is observed at $55 \pm 3^\circ\text{C}$ (data not shown). Even at a temperature of 95°C a considerable amount of secondary structure still exists (**Fig 7**). Upon annealing to 20°C rHom s 2.01 essentially regains its initial secondary structure.

rHom s 2 induces specific lymphoproliferation Two AD patients containing comparable total serum IgE levels, one with (**Table I**, **Fig 8**: patient 1) and one without Hom s 2-specific IgE (**Table I**, **Fig 8**: patient 2) were studied by lymphoproliferation assays. In two independently performed experiments similar results were obtained: different concentrations of rHom s 2 ($0.6 \mu\text{g}$ per well, $2.5 \mu\text{g}$ per well) induced specific lymphoproliferative responses (stimulation indices > 2.5) in PBMC of the rHom s 2-reactive (patient 1) but not in the other AD patient (patient 2) without Hom s 2-specific IgE (**Fig 8**). Only the AD patient 2 with IgE antibodies against the dog allergen, Can f 3, but not AD patient 1 without Can f 3-specific IgE showed proliferative responses (stimulation indices > 2.5) to Can f 3 (**Fig 8**). PBMC of both AD patients showed proliferation (SI between 5 and 7) after addition of interleukin-2. **Table I** displays the IgE and IgG subclass reactivity of the two AD patients to rHom s 2 and Can f 3 as determined by enzyme-linked immunosorbent assay (Vrtala *et al*, 1996). As observed previously for environmental allergens, IgE reactivity to Hom s 2 was not strictly associated with IgG subclass reactivity (Valenta and Ball, 1997).

DISCUSSION

Using serum IgE from an AD patient we have previously isolated a partial cDNA clone coding for the 86 C-terminal amino acids of human α -NAC, a component of a heterodimeric protein complex, NAC, which is required for signal-sequence-specific sorting and translocation of nascent polypeptides emerging from the ribosomes

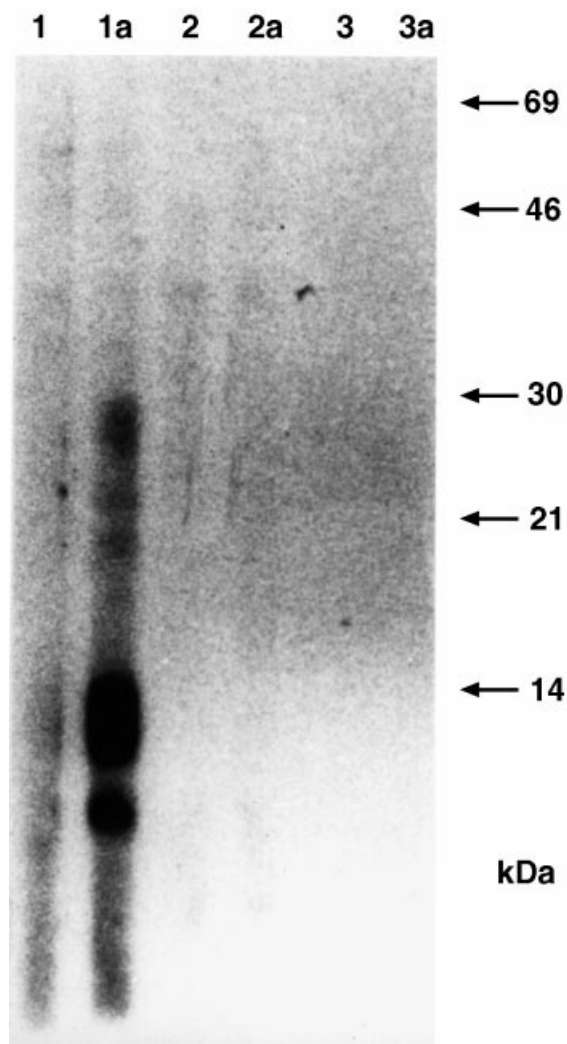


Figure 6. rHom s 2.01 inhibits IgE binding to its natural counterpart. Nitrocellulose-blotted human colon extract was incubated with serum from an AD patient with (lane 1) and without (lane 2) rHom s 2.01-reactive IgE as well as with buffer alone (lane 3). In the first lane, sera were preadsorbed with *E. coli* extract containing rHom s 2.01 and in lane a with *E. coli* extract alone. Bound IgE antibodies were detected with ^{125} I-labeled anti-human IgE antibodies and visualized by autoradiography. Molecular weights (kDa) are indicated on the right margin.

in human and other mammalian cells (Wiedmann *et al*, 1994; Natter *et al*, 1998). In addition murine α -NAC has been also identified as a transcriptional coactivator (Yotov and St Arnaud, 1996).

In this study we isolated by oligonucleotide screening of a human epithelial cDNA library a cDNA coding for a complete human α -NAC isoform, which differs in 21 of 215 amino acids from the previously described human α -NAC form. Further support for our assumption that several α -NAC isoforms exist in humans was obtained by Southern blot experiments where we demonstrated that digestion of human genomic DNA with enzymes that do not cut in the α -NAC-encoding cDNA yielded several bands capable of hybridizing with the α -NAC cDNA probe.

Although α -NAC represents a phylogenetically conserved protein with important biologic functions, there is to date no report regarding the molecular characterization of a folded

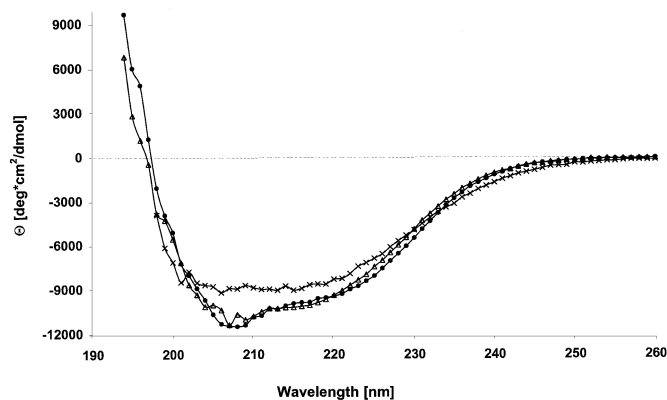


Figure 7. Circular dichroism analysis of purified rHom s 2.01 at various temperatures. The plot of the mean residue molar ellipticity shows a mixed α / β -fold at 20°C (full dots). A slight unfolding can be observed at 95°C (crosses), but after re-annealing to 20°C (open triangles) the initial secondary structure is mostly regained.

Table I. IgE and IgG subclass reactivity to rHom s 2.01.

Optical density values corresponding to the levels of IgE and IgG₁–4 antibodies specific for rHom s 2 and the dog allergen Can f 3 are displayed for sera from the two AD patients (1, 2) which were analyzed for Hom s 2-specific lymphoproliferative responses in Fig 8

		rHom s 2	Can d 3
No. 1	IgE	0.228	0
	IgG ₁	0.041	0
	IgG ₂	0	0
	IgG ₃	0	0
	IgG ₄	0.066	0
No. 2	IgE	0	0.961
	IgG ₁	0.015	0.198
	IgG ₂	0.054	0.031
	IgG ₃	0	0
	IgG ₄	0.024	0.510

recombinant α -NAC protein. In order to obtain a folded recombinant α -NAC protein as a paradigmatic tool for immunologic experiments we used the cDNA coding for the human α -NAC isoform isolated in our study to express recombinant human α -NAC as His-tagged protein in *E. coli*. Using nickel affinity chromatography we were able to purify milligram amounts of soluble recombinant human α -NAC. Circular dichroism analysis demonstrated that recombinant α -NAC represented a folded protein consisting of mixed α -helical and β -sheet conformation and exhibited a remarkable thermal stability and refolding capacity. This feature has been reported for chaperone-like proteins and seems to be common to many important environmental allergens (Laffer *et al*, 1996; Hayek *et al*, 1998; Mittermann *et al*, 1998; DeMarino *et al*, 1999; Niederberger *et al*, 1999; Vrtala *et al*, 1999; Bugajska-Schretter *et al*, 2000).

Using recombinant α -NAC we were able to document its immunologic equivalence with natural α -NAC by IgE inhibition experiments and demonstrate the specific reactivity of recombinant α -NAC with IgE autoantibodies. The recombinant α -NAC isoform described in our study was therefore designated Hom s 2.01 according to the Allergen Nomenclature System. It may be used to identify atopic patients containing IgE autoantibodies against α -NAC. The latter assumption is supported by our finding that rHom s 2.01 such as environmental allergens, can be used in enzyme-linked immunosorbent assay assays to identify atopic

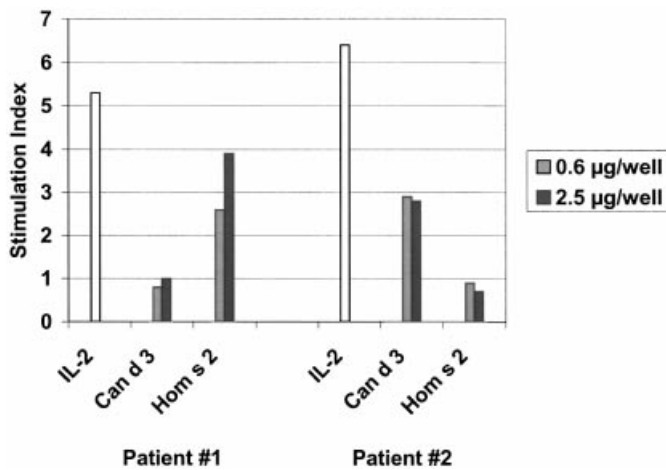


Figure 8. rHom s 2 induces specific lymphoproliferative responses. PBMC from two AD patients, one with (patient 1) and one without (patient 2) Hom s 2-specific IgE were stimulated with different concentrations of rHom s 2 and the dog allergen Can f 3 as well as with interleukin-2 (x-axis). Stimulation indices are displayed on the y-axis.

patients containing specific IgE autoantibodies. In this context it was of note that the C-terminus of Hom s 2, which represented the most conserved portion of α -NAC-homologous proteins from animals, insects, yeast, plants, and protozoa, contained binding sites for IgE autoantibodies. At present, however, we do not have a definitive answer to the question whether α -NAC-reactive patients were originally sensitized against an α -NAC-homologous protein from plants, yeast, or insects and then started to exhibit IgE cross-reactivity to endogenous α -NAC or whether they were primarily sensitized against endogenous α -NAC.

AD represents a chronic skin manifestation of atopy and it is well established that T cell reactivity to exogenous allergens represents a prominent feature of AD (Rawle *et al*, 1984). These T cells can be found in the circulation of AD patients but also home to the skin where they can give rise to positive atopy patch test reactions (Van Reijssen *et al*, 1992; Santamaria-Babi *et al*, 1995; Wistokat-Wulfing *et al*, 1999). Using rHom s 2 we show that AD patients containing IgE autoantibodies can also exhibit specific lymphoproliferative responses to IgE-defined autoantigens. It is thus possible that T cell reactivity to autoantigens, such as T cell reactivity to exogenous allergens, may represent a pathomechanism in AD. Although only very few patients (approximately 1% of AD patients) contained α -NAC-specific IgE autoantibodies, we think that recombinant α -NAC may serve also as a paradigmatic tool to study further mechanisms of IgE autosensitization, e.g., by establishing experimental animal models for IgE autoreactivity (Herz *et al*, 1997; Neuhaus-Steinmetz *et al*, 2000).

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