

Hom s 4, an IgE-Reactive Autoantigen Belonging to a New Subfamily of Calcium-Binding Proteins, Can Induce Th Cell Type 1-Mediated Autoreactivity¹

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Skin inflammation in atopic dermatitis starts with Th2 and IgE-mediated responses against exogenous allergens and, for unknown reasons, resembles features of a Th1-driven reaction in the chronic stages. We report the characterization of a human protein, Hom s 4, recognized by IgE autoantibodies from atopic dermatitis patients. The complete Hom s 4 cDNA codes for a 54-kDa basic protein containing two typical calcium-binding domains separated by an unusually long α -helical domain. Therefore, Hom s 4 and homologous proteins found by sequence comparison in mice, fruit flies, and nematodes constitute a novel subfamily of calcium-binding proteins. Using Hom s 4-specific Abs, it is demonstrated that the protein is strongly expressed within epidermal keratinocytes and dermal endothelial cells. Purified Hom s 4 showed IgE cross-reactivity with exogenous calcium-binding allergens from plants and fish but, in contrast to the exogenous allergens, induced only weak histamine release from patient basophils. However, the analysis of Hom s 4-specific cytokine and humoral immune responses indicated that Hom s 4 strongly induces Th1 responses which are accompanied by the release of IFN- γ , a cytokine implicated in epithelial cell damage. Hom s 4-induced IFN- γ production was found in normal individuals, in patients with chronic inflammatory skin diseases and in Th2-prone atopic persons, suggesting that Hom s 4 represents a protein with an intrinsic property to induce Th1-mediated autoreactivity. It may thus contribute to chronic skin inflammation in atopic as well as in nonatopic persons. *The Journal of Immunology*, 2005, 175: 1286–1294.

Atopic individuals are characterized by their disposition to mount Th2-dominated cellular and IgE Ab responses against per se harmless Ags (i.e., allergens) (1). A great variety of factors, including environmental conditions, genetic factors, and modes of allergen contact, influence the magnitude and specificity of the emerging allergic immune response (2, 3). Depending on the type of immune cells involved in allergic inflammation, immediate and late reactions may occur. Cross-linking of effector cell (i.e., mast cell, basophil)-bound IgE leads within few minutes to the release of inflammatory mediators (e.g., histamine, leukotrienes) and thus to the acute symptoms of allergic disease (4). Chronic symptoms are dominated by the activation of allergen-specific T cells and by the subsequent influx of eosinophils, a process that can be enhanced by IgE-mediated Ag presentation (5, 6).

Acute and chronic manifestations of allergy may occur isolated or simultaneously in different target organs, thus leading to a pleth-

ora of disease phenotypes (e.g., rhinoconjunctivitis, food allergy, asthma, atopic dermatitis, anaphylactic shock). Atopic dermatitis (AD),³ a chronic, eczematous skin manifestation of atopy affecting almost 10% of children, mainly resembles features of a T cell-mediated immune reaction (7–9). The importance of T cell-mediated immune reactions in AD is underlined by several findings. First, it has been demonstrated that AD patients show stronger lymphoproliferative responses to environmental allergens than patients suffering from allergic rhinoconjunctivitis (RC) or asthma (10). Second, T cells specific for respiratory and food allergens have been detected in the blood of AD patients that express skin-selective homing receptors and thus migrate into the skin, especially after local allergen exposure (11, 12). Furthermore, allergen-specific memory T cells can be identified via their hypervariable regions in the skin of AD patients for years (13). Third, T cell-mediated induction of keratinocyte apoptosis has been suggested as a pathogenetic mechanism operative in AD (14). Finally, evidence for T cell involvement in the pathogenesis of AD comes from the finding that drugs acting preferentially on T cells (e.g., cyclosporin, FK506) are effective in the treatment of AD (15). A link between T cell and IgE Ab-related effects could be established by the finding that IgE-mediated presentation of allergens can lead to a strong enhancement of allergen-specific T cell responses and the subsequent release of proinflammatory cytokines (16, 17). In fact, AD patients frequently exhibit strongly elevated levels of serum IgE Abs against a variety of environmental allergens from various sources (e.g., pollen, mites, animal dander, food, molds). It

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³ Abbreviations used in this paper: AD, atopic dermatitis; HSA, human serum albumin; CBMC, cord blood mononuclear cells; RC, allergic rhinoconjunctivitis; NA, nonatopic individuals; CD, contact dermatitis; PS, psoriasis.

has also been shown that exogenous allergen contact can lead to exacerbation of skin manifestations (18). On the other hand, evidence exists that >60% of AD patients mount IgE Ab responses against endogenous human proteins (19). In this context, it was found that the intensity of IgE autoreactivity is associated with the severity of skin manifestations (19–21). Using IgE from AD patients, cDNAs coding for IgE-reactive autoantigens have been isolated, and their molecular nature has been revealed (20, 22, 23). Here we report the molecular characterization and expression in *Escherichia coli* of one of the previously characterized five IgE-reactive autoantigens, designated Hom s 4. It was recognized by ~10% of AD patients containing IgE autoantibodies and represented a protein with as yet unknown biological function. The unexpected finding that Hom s 4 has the intrinsic property to induce the release of the Th1 cytokine IFN- γ is discussed regarding two aspects. Regarding AD, we suggest that reactivity to IFN- γ -driving autoantigens may contribute to IFN- γ -mediated tissue damage in the chronic phase of AD. The finding that persons without a Th2-biased immune response (e.g., nonatopic persons, patients with chronic inflammatory skin diseases) mounted even stronger IFN- γ responses to Hom s 4 suggests that it may contribute also to other nonatopic chronic inflammatory skin diseases.

Materials and Methods

Characterization of individuals and patients

Sera were obtained from 23 atopic patients (AD, $n = 12$; RC without AD, $n = 11$), 6 nonatopic individuals, and 6 nonatopic (NA) individuals suffering from chronic dermatoses (contact dermatitis, $n = 3$; psoriasis, $n = 3$). AD patients were characterized according to the criteria of Hanifin and Rajka (24). Demographic data and clinical and laboratory features of these individuals (21 men, 14 women; ages 20–72 years; average age, 38.5 years) are summarized in Table I.

The diagnosis of IgE-mediated allergy was based on case history and the demonstration of serum IgE Abs against common environmental allergens. Total serum IgE levels were determined by CAP System FEIA measurements (Pharmacia Diagnostics). IgE autoantibodies were detected in sera using nitrocellulose-blotted extracts of the human epithelial cell line A431 as described (19).

Cell lines, protein extracts, cell fractionation, and organelle isolation

The human epithelial cell line A431 was purchased from American Type Culture Collection. Cellular pellets containing $\sim 5 \times 10^6$ cells were resuspended in 1 ml of SDS sample buffer, boiled for 10 min, and subjected to denaturing 12.5% preparative SDS-PAGE (25). Protein extracts were then blotted onto nitrocellulose membranes (Schleicher & Schuell) (26).

A431-derived cellular subfractions (cytoplasm) and organelles (mitochondria, nuclei) were prepared according to the method of differential centrifugation by velocity (27, 28).

E. coli strains, plasmids

The coding region of the Hom s 4 cDNA (20) was amplified by PCR using a platinum PCR Supermix kit (Life Technologies) with the following primer pair: CALC 1, 5'-GCT CTA GAA ATA ATT TTG TTT AAC TTT AAG AAG GAG ATA TAC ATA TGT TTC GTC TGA ACT CAC TTT C 3' and CALC 2, 5'-GGA ATT CCG AGT GGT GGT GGT GGT GGT GCT GTT TGG GTA AAG CGA AGT CCC-3' (MWG Biotech). The plasmid clone DKFZp564C246 obtained from the German Human Genome Project served as a template (Fig. 1). The *Xba*I and *Eco*RI restriction sites are underlined and printed in italics, respectively. A sequence coding for a hexahistidine tag (bold) was incorporated into the CALC 2 primer. Cycling conditions were: 94°C, 60 s; 55°C, 60 s; and 72°C, 60 s for 35 cycles, followed by a terminal extension cycle at 72°C for 10 min.

The PCR product was digested with *Eco*RI and *Xba*I (Roche Diagnostics), gel-purified and ligated into an *Eco*RI/*Xba*I-restricted pET-17b plasmid (Novagen). The cDNA sequence of the pET-17b-Hom s 4 construct was confirmed by automated DNA sequencing (MWG Biotech). The amino acid sequence of Hom s 4 was established using the McVector program (Kodak). Molecular mass and the isoelectric point of Hom s 4 as well as its secondary structure and sequence motifs were also analyzed with the McVector program. The cDNA and deduced amino acid sequence of Hom s 4 were compared with the sequences deposited in GenBank using the BLAST program.

Expression and purification of recombinant Hom s 4

Escherichia coli strain BL21 (DE3): F⁻, *ompT* r_b-m_b⁻ (DE3) was transformed with the expression plasmid pET-17b-Hom s 4 and grown in Luria-Bertani medium containing 100 mg/L ampicillin at 37°C to an OD_{600 nm} of 0.4. Protein expression was induced by addition of isopropyl- β -D-thiogalactopyranoside to a final concentration of 0.5 mM and further growing of cells for additional 4 h. *E. coli* cells were then harvested by centrifugation at $8000 \times g$ at 4°C for 15 min.

Because rHom s 4 accumulated in the insoluble inclusion body fraction of the bacteria, the protein was purified by inclusion body preparation, solubilization, and nickel chelate affinity chromatography (Qiagen).

Cells were homogenized in 25 mmol/L imidazole (pH 7.4) containing 0.1% v/v Triton X-100 and lysozyme (50 μ g/g cells). The homogenate was incubated with DNase I (100 μ g/g cells) for 20 min at room temperature. After centrifugation ($15,000 \times g$ for 20 min at 4°C), the pellet was resuspended in buffer P (20 mmol/L Tris-HCl (pH 8.0), 200 mmol/L NaCl, 1% w/v deoxycholate, and 1 mmol/L 2-ME). The homogenate was centrifuged again, and the inclusion body pellet was washed four times with 4-fold diluted buffer P and finally with 10 mmol/L Tris-HCl, pH 8.0, containing 3% v/v isopropanol. Inclusion bodies were solubilized in 8 mol/L urea, 0.1 mol/L NaH₂PO₄, 0.01 mol/L Tris-HCl, pH 8.0. Insoluble material was removed by centrifugation at $15,000 \times g$ (20 min, 4°C), and the rHom s 4-containing supernatant was applied to nickel-nitrilotriacetic acid-Sepharose (Qiagen). The nickel-nitrilotriacetic acid-Sepharose column was washed with 50 ml of buffer C (8 mol/L urea, 0.1 mol/L NaH₂PO₄, 0.01 mol/L Tris-HCl, pH 6.3). rHom s 4 was eluted with elution buffer E (8 mol/L urea, 0.1 mol/L NaH₂PO₄, 0.01 mol/L Tris-HCl, pH 4.5). Fractions containing eluted rHom s 4 were pooled and dialyzed stepwise against 0.1 mol/L NaH₂PO₄; against 0.01 mol/L Tris-HCl (pH 7.4), containing 6, 4, and 2 mol/L urea; and finally against PBS. The protein concentration was determined by Micro BCA Protein Assay (Pierce). The purity and integrity of rHom s 4 were assessed by SDS-PAGE (25) and subsequent staining with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) (29). In addition, the rHom s 4 preparation was blotted onto nitrocellulose (26) and tested for reactivity with a monoclonal anti-His-tag Ab (Novagen) as described (30).

Analysis of specific IgE reactivity

The specific IgE binding capacity of rHom s 4 was tested by IgE immunoblotting. Nitrocellulose-blotted rHom s 4 was exposed to sera from atopic patients with and without Hom s 4-specific IgE, serum from a nonatopic person, or buffer alone, and bound IgE was detected as described (31). In addition, the IgE reactivity of rHom s 4 was tested in nondenaturing dot-blot assays. For this purpose, 1- μ l aliquots containing 1 μ g of recombinant Hom s 4, 1 μ g of the recombinant timothy grass pollen allergens rPhl p 7, rPhl p 2, and rPhl p 5 (Biomay), 1 μ g of the synthetic peptides representing the N-terminal (aa 1–37) and C-terminal (aa 36–78) portion of rPhl p 7, and 1 μ g of human serum albumin (HSA; Behringwerke), were dotted onto nitrocellulose membranes (Schleicher & Schuell) and exposed to sera or buffer as described for immunoblotting (31).

IgE cross-inhibition and Ca²⁺ depletion experiments

The presence of potential cross-reactive IgE epitopes between rHom s 4 and exogenous calcium-binding allergens (rPhl p 7 from timothy grass pollen (32, 33); rCyp c 1 from fish (34)) was studied by dot-blot inhibition studies. Nitrocellulose membranes containing a set of dotted allergens (1 μ g/dot of rHom s 4, rCyp c 1, rPhl p 7) and HSA (1 μ g/dot) were exposed to serum samples from Hom s 4-reactive AD patients which had been preincubated individually with each of these proteins (5 μ g/ml in a 1/10 serum dilution). Bound IgE Abs were detected as described (31).

The effect of depletion of protein-bound calcium (Ca²⁺) ions on the IgE-binding capacity of rHom s 4 and the recombinant timothy grass pollen allergens rPhl p 7, rPhl p 2, and rPhl p 5 was investigated by dot-blot studies. Serum samples from Hom s 4-reactive AD patients including EGTA (2 mmol/L) or CaCl₂ (0.1 mmol/L) were incubated with a set of nitrocellulose-dotted proteins (1 μ g/dot of rHom s 4, HSA, rPhl p 2, rPhl p 5, and rPhl p 7). Bound IgE Abs were detected as described (31).

Hom s 4 gene expression in different organ specimens

Human tissue samples were obtained from biopsies either taken ex vivo (bone marrow, placenta, skin) or immediately post mortem (lung, liver, colon). Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Reverse transcription PCR were performed using the Protoscript First Strand cDNA Synthesis kit (New England Biolabs) using 1 μ g of total RNA in a total volume of 50 μ l. PCR conditions

Table I. Clinical and demographic characterization of individuals^a

	<i>n</i> = 35	F:M	Age (yr)/Mean	IgE Autoreactivity (%)	rHom s 4 (%)	rPhl p 7 (%)	rPhl p 1 (%)	total IgE (kU/L)/Mean
AD	12	5:7	20–72/38.4	91.7	16.7	16.7	83.3	190–5000/2307.4
RC	11	3:8	21–65/37.9	0	0	45.5	90.9	18–1324/292.4
NA	6	2:4	27–42/35.2	0	0	0	0	2–148/33.8
CD	3	2:1	44–52/47.0	0	0	0	0	12–26/19.7
PS	3	2:1	29–53/39.7	0	0	0	0	6–8/7.0

^a Gender, age, type of disease, and total IgE levels (kU/L) of 23 atopic patients (AD, *n* = 12; RC without AD, *n* = 11), 6 NA individuals, and six individuals suffering from chronic dermatoses (CD, *n* = 3; PS, *n* = 3) are displayed. The percentage of individuals with IgE reactivity to blotted human epithelial cell extracts (IgE autoreactivity), rHom s 4, rPhl p 7, and rPhl p 1 is indicated.

were initial denaturation 94°C for 60 s, annealing at 55°C for 60 s, polymerization at 72°C for 60 s (35 cycles), and terminal extension at 72°C for 10 min. To determine the tissue expression of Hom s 4, the Hom s 4 PCR was conducted together with a β -actin PCR in the same tube as double-product semiquantitative PCR. The following primer pairs were used: β -actin forward 5'-ATG GAT GAT GAT ATC GCC GCG-3' and β -actin reverse 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG GCC-3'; Hom s 4 forward 5'-ATG TTT CGT CTG AAC TCA CT-3' and Hom s 4 reverse 5'-CCC TTC ATC TTT ATT CTT CC-3'. The products were resolved in 1.5% agarose gels containing 0.5 μ g/ml ethidium bromide.

Immunohistochemistry of rHom s 4

Skin punch biopsies from AD patients and healthy individuals were embedded in Tissue-Tec II (Miles) and frozen in liquid nitrogen. Cryostat sections (5 μ m) were fixed for 10 min in acetone at 4°C and subjected to immunostaining. After washing with PBS, sections were incubated with equal concentrations of rabbit anti-Hom s 4 Ig or preimmune Ig for 1 h at room temperature (21). Primary Ab binding was visualized using biotinylated donkey anti-rabbit (Fab')₂ (Amersham) and the Vectastain Elite ABC KIT (Vector Laboratories). Sections were counterstained with hematoxylin (Merck).

FIGURE 1. Sequence comparison of rHom s 4. The Hom s 4 amino acid sequence (Accession No. AL117423) (top line) was aligned with homologous proteins from mouse (*M. musculus*, Accession No. AAH26566), fruit fly (*D. melanogaster*, Accession No. NP_609100) and free living soil nematode (*C. elegans*, Accession No. CAC44305). Identical amino acids are indicated by dashes; points indicate gaps. The two calcium-binding domains are underlined; arrow, beginning of the original IgE-reactive fragment (20).

Hom s 4 #AAH26566 #NP_609100 #CAC44305	MFR...LNSLSALAE MSVLRFLVTRQALAAALT--IIQNP-Q MQTRSVDKSTASRETAHCKAPNFIHFLMLHCSFLRVIPIKNASKRLIIVRS-T-AP-KTS
Hom s 4 #AAH26566 #NP_609100 #CAC44305	LAVGSRWY.....HGGSQPIQIRRLMMVAFLGAS.....AVTASTG..... -----A---T-TK-----L----- I-YA-TLCNQNSN-NAKDLTKSSAN-SLMQTR-HKRFHQEEK-P-VTKYFHMFLSLFL E-EKQDQSLKNIDA-EKYTALGNI-QKKDV-HYTRDRAGSVGYSHY-SSVYQHRPIYWPL
Hom s 4 #AAH26566 #NP_609100 #CAC44305	...LLWKGAHAESPCCVDNLK...SDIGDKGNKDEGDVCNHEKKTADLA..PHPEEKK ...-K-----NSK-...P-TE--ER--S-E-SSR-GRA--A-AE---D- ISVMD-GKVKRMLT-K--ADAGQRP-SAA-VNGEDKSSSESEDEEAGSDL-LH-G- RKLYH-NY-LVIAGMVILMSDFEWLK-QIKSASLPFRPEASQK-EV-ESNGEVEEVK--P
Hom s 4 #AAH26566 #NP_609100 #CAC44305	KKRS..GFRDRKVMYENRIRAYSTPDKIFRYFATLKVIS...EPGEAEVFMTPEDFVRS-N...-T-----Q----- -I-EKV---E---II-----QF-----VRLQD...AT.QTI-C-----L-- --KK-L---E-RII---D-L-L-----I-DPNEDS-RF-----L--
Hom s 4 #AAH26566 #NP_609100 #CAC44305	ITPNEKQPEHLGLDQYIIKRFDGKTEKISQEREKFADEGSIFYTLGECGLISFSDYIFLT -----A-----S----- -YPGIK-DG-----R-Y-P...SVGEQLNLHLEKN--K--SY--T-----L F--GVM-RRW---SF---NYNPE...-KHK-H--S-PD--K--N--N--L--M
Hom s 4 #AAH26566 #NP_609100 #CAC44305	TVLSTPQRNFEIAFKMFDLNGDGEVDMEEFEQVQSIIIRSQTSMGMRHRDRPTTGNTLKS ---ISR-H---R-----D-C---M-ATLV-Q---T---HAN---F--- -L---SHAD-AL---I--VD-N-AL-K---TK--QL-M---TV-Q---HI-PNQSFVE
Hom s 4 #AAH26566 #NP_609100 #CAC44305	LCSALTTYFFGADLKGKLTIKNFLEFQRLQHDVLKLEFERHDPVD...GRITERQFGGM -----S----- V---I---PNMDE---EK--D--EQ--REI-S---KE-NDE..N--AD-AEL TN---E---K-G--S-SSEK-I---ER---I--M---R-AL-NPD-L-N-DS-AQL
Hom s 4 #AAH26566 #NP_609100 #CAC44305	LLAYSGVQSKKLTAMQRQLKKHFKE..GKGLTFQEVENFFTLKNINDVDTALSFYHMA -----D..... ---A-YPL--KQKKLKRVR--RDH---ISK-DYLD--H--N-----T---I-- --LHAQINE--QKH-LKRVR--GENL--IS-G-TKA--E--YH-D---I--H-HK--
Hom s 4 #AAH26566 #NP_609100 #CAC44305	ASLDKVTMQQVARTVAKVELSDHVCDDVFALFDGNGELSNKEFVSIMKQRLMRGLEKP --I-QQ-L-H--K--M-N---V---TI--ENNDNQ-----I-V--N-VQ----- M-I-AKLL-R--VK-TGIP-----V---IT---DNLD-K--HE-M-AV-RR-MR---R-
Hom s 4 #AAH26566 #NP_609100 #CAC44305	KDMGFTRLQAMWKAQETAWDFALPKQ ----- --T--LKM-RSVF--K--KPVLLDI.. R-T-LFR-FD-VLE-GKRAYHASP--FY

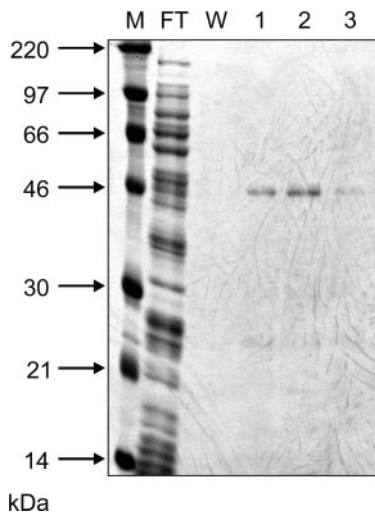


FIGURE 2. Expression and purification of His-tagged recombinant Hom s 4 via nickel affinity chromatography. A molecular mass marker (lane M), the flow-through (lane FT), the wash solution (lane W), and the eluted fractions (lanes 1–3) were separated by SDS-PAGE and stained with Coomassie blue. Molecular masses (in kilodaltons) are indicated on the left margin.

Detection of Hom s 4 in subcellular fractions and in tissues

A rabbit anti-rHom s 4 antiserum was raised by immunizing a rabbit with purified rHom s 4 using Freund's adjuvant (Charles River Laboratories). Nitrocellulose-blotted subcellular fractions (mitochondria, cytoplasm, microsomes, and nuclei) prepared from the human epithelial cell line A431 were exposed to the rabbit anti-Hom s 4 antiserum or the rabbit's preimmune serum, each diluted 1/1000. Bound rabbit Abs were detected with a ^{125} I-labeled donkey anti-rabbit antiserum (Amersham) as described (31). The successful preparation of the nuclear subfraction was confirmed with a rabbit anti-human histone H2B antiserum (Chemicon) as described (28).

Basophil histamine release experiments

Granulocytes were obtained from the peripheral blood of two AD patients with rHom s 4-specific IgE Abs and T cell reactivity. Granulocytes were prepared by dextran sedimentation (35) and exposed to various concentrations of rHom s 4 or the grass pollen allergen, Phl p 7. Histamine released into the culture supernatants was determined by radioimmunoassay (Immunotech) as described (35). All experiments were performed in triplicates and results represent mean values.

Lymphoproliferative assays, detection of Ag-specific cytokine, and Ab levels

Heparinized venous blood samples were collected from atopic patients as well as from nonatopic individuals after informed consent was given. Human umbilical cord blood from four full term healthy infants (>37 wk of gestation) was obtained by venipuncture of the umbilical vein immediately

after delivery and placed in sterile sodium heparin tubes. PBMC as well as cord blood mononuclear cells (CBMC) were isolated by Ficoll (Amersham Pharmacia Biotech) density gradient centrifugation. PBMC (2×10^5) were cultured in triplicates in 96-well plates (Nunc) in serum-free Ultra Culture medium (BioWhittaker) supplemented with 2 mM L-glutamine (Sigma-Aldrich), 50 μ M β -ME (Sigma-Aldrich), and 0.1 mg/ml gentamicin (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were stimulated with different concentrations (0.6, 1.25, 2.5, and 5 μ g/well) of the timothy grass pollen allergens rPhl p 1 (36) and rPhl p 7 (32, 33), rHom s 4, and, for control purposes, with 4 U of IL-2/well (Boehringer-Mannheim), tetanus toxoid (0.2 μ g/well) (Calbiochem), or medium alone in triplicates.

Endotoxin concentrations (measured in endotoxin U (EU)/ml) were determined in all of the tested Ag preparations using *Limulus* Amebocyte Lysate QCL-1000 (BioWhittaker) according to the manufacturers' instructions. (rHom s 4, 0.2 μ g, 0.066 EU/ml; rPhl p 7, 0.2 μ g, 0.057 EU/ml; rPhl p 1, 0.2 μ g, 0.131 EU/ml).

T cell proliferation was measured by [^{3} H]thymidine uptake as described (37). The stimulation indices were calculated as the quotient of the mean cpm values of the stimulated (IL-2) and the unstimulated medium control. Supernatants of PBMC cultured with purified allergens (2.5 μ g/ml) were harvested at day 6 of culture. The levels of IL-4, IL-5, IL-10, and IFN- γ released into the supernatants were measured by ELISA using matched Ab pairs (Endogen) according to the manufacturer's instructions (sensitivity limits: IL-4, 4 pg/ml; IL-5, 3.9 pg/ml; IFN- γ , 8.1 pg/ml; IL-10, 3.3 pg/ml).

For ELISA detection of rHom s 4-, rPhl p 7- and rPhl p 1-specific IgE and IgG subclass responses, plates (Maxisorb; Nunc) were coated with 5 μ g/ml purified rHom s 4, 5 μ g/ml purified rPhl p 7, or 5 μ g/ml purified rPhl p 1 (Biomay). Specific IgE and IgG₁₋₄ subclass responses were detected as described (38). Statistical differences were analyzed using the paired Student *t* test. Significant differences ($p < 0.05$) are indicated by one asterisk and highly significant differences ($p < 0.01$) are indicated by two asterisks.

Results

Hom s 4 belongs to a novel subfamily of calcium-binding proteins

Using serum IgE from atopic dermatitis patients, we have isolated cDNAs coding for IgE-reactive autoantigens (20). One of these IgE-reactive cDNA clones coded for a C-terminal fragment of a calcium-binding protein which was designated Hom s 4. The complete cDNA of Hom s 4 (1437 nucleotides including start and stop codon) codes for a protein of 478 amino acids with a calculated molecular mass of 54.2 kDa and a predicted isoelectric point of 8.67. The deduced amino acid sequence of Hom s 4 is characterized by the presence of only four tryptophan residues and a relatively high number of lysine residues (8.6%). Secondary structure prediction indicates that the protein consists almost exclusively of α helices. A search for sequence motifs revealed the presence of two classical calcium-binding motifs (Fig. 1, underlined) which are separated by an unusually long α -helical spacer.

A database search for Hom s 4-related proteins identified homologous proteins in the mouse (*Mus musculus*), fruit fly (*Drosophila melanogaster*), and a nematode (*Caenorhabditis elegans*) (Fig. 1). Hom s 4 and the three related proteins exhibited a similar architecture (two calcium-binding domains separated by a long spacer) and thus may be considered as a novel subfamily of calcium-binding proteins.

The highest degree of sequence identity (93%) was found between Hom s 4 and the corresponding protein from the mouse. Lower sequence identities (50 and 44%, respectively) were found between Hom s 4 and the homologous proteins from the fruit fly and the free-living soil nematode.

Purification of rHom s 4

rHom s 4 was isolated by inclusion body preparation from induced *E. coli* cells and purified by nickel affinity chromatography. The Coomassie-stained SDS-PAGE (Fig. 2) shows that rHom s 4 can be eluted from the affinity column under acidic conditions as a

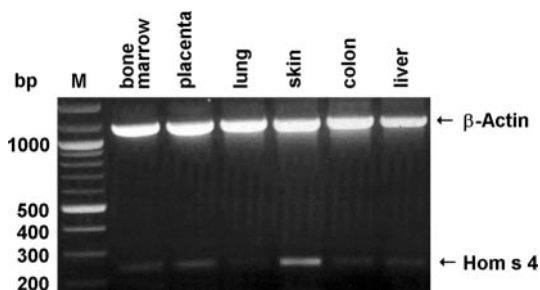
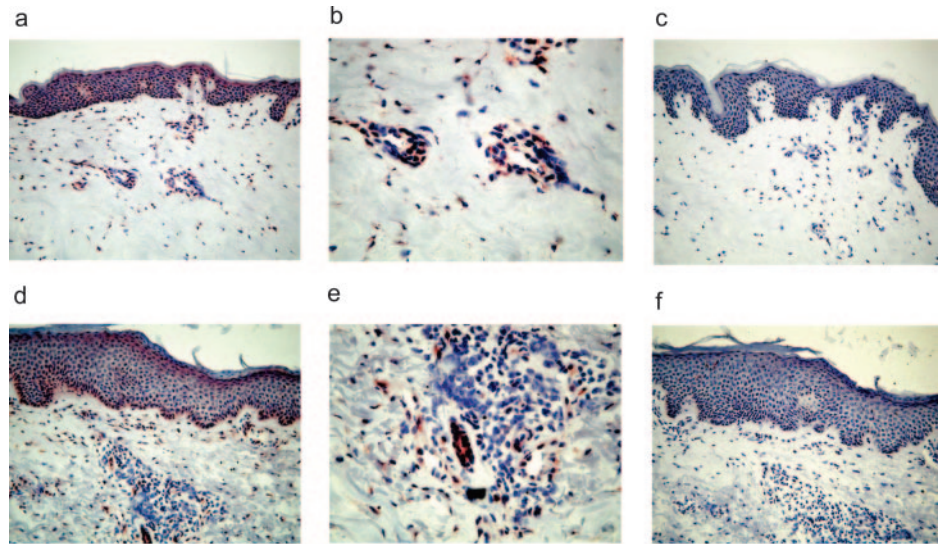


FIGURE 3. Hom s 4 gene expression in different human organ specimens. Reverse transcription PCR were conducted with RNA samples from various organs. The PCR products (β -actin, Hom s 4) were resolved in 1.5% agarose gels. Lane M shows the molecular masses in base pairs.

FIGURE 4. Immunohistological localization of Hom s 4 in human skin sections. Cryostat sections of normal skin (*a–c*) and AD skin (*d–f*) were stained with rabbit anti-Hom s 4 Ig (*a, b, d, and e*) or preimmune rabbit Ig (*c and f*). Note the prominent cytoplasmic staining of epidermal cells (*a and d*) and endothelial cells (*b and e*), and slightly weaker reactivity with dermal nerves.



protein with an apparent molecular mass of ~ 46 kDa. Weakly visible coeluting bands with smaller and occasionally larger molecular masses than the 46 kDa band could be identified as rHom s 4-derived by their reactivity with a monoclonal anti-His-tag Ab and serum IgE from Hom s 4-reactive AD patients (data not shown). The rHom s 4 preparations used for the cellular experiments contained similar or lower levels of endotoxins than the recombinant grass pollen allergens (rHom s 4, 0.2 μ g, 0.066 EU/ml; rPhl p 7, 0.2 μ g, 0.057 EU/ml; rPhl p 1, 0.2 μ g, 0.131 EU/ml).

Hom s 4 is predominantly expressed in skin and preferentially occurs in mitochondrial and microsomal subcellular fractions

RT-PCR showed that the Hom s 4 gene is most strongly expressed in the skin and, to a much lower degree, in various other organs (bone marrow, placenta, lung, colon, and liver) (Fig. 3). Exposure of human skin sections from atopic dermatitis patients as well as from healthy individuals to rabbit anti-Hom s 4 Abs revealed strong cytoplasmic reactivity with epidermal keratinocytes (Fig. 4, *a and d*) and dermal endothelial cells (Fig. 4, *b and e*) and a weaker staining of dermal nerves (Fig. 4, *a, b, d, and e*). No immune reactivity was found when skin sections were incubated with pre-immune Ig (Fig. 4, *c and f*).

The subcellular distribution of Hom s 4 was analyzed in the human epithelial cell line A431 (Fig. 5). Rabbit anti-rHom s 4 Abs (*lanes b*), but not the rabbit's preimmune Ig (*lanes a*) reacted with a 46 kDa band in nitrocellulose-blotted microsomal > mitochondrial > cytoplasmic subcellular fractions (Fig. 5). No specific immune reactivity was found in the nuclear fraction (Fig. 5, *panel 4*).

IgE reactivity to rHom s 4 is calcium dependent

We found that $\sim 10\%$ of 100 AD patients contain IgE autoantibodies against rHom s 4 (data not shown). As exemplified in Fig. 6a, these patients showed also IgE reactivity to a highly cross-reactive calcium-binding allergen from grass pollen, Phl p 7 (32, 33) (Fig. 6a, patients 1 and 2). Phl p 7-reactive patients with respiratory allergy but without AD (patients 3–7) showed no IgE reactivity to rHom s 4 (Fig. 6a). The specificity of the IgE reactivity to Hom s 4 was confirmed by the fact that AD patients with highly elevated total IgE levels (Fig. 6a, patients 8–10) showed no IgE binding to rHom s 4.

The IgE epitopes of the calcium-binding allergen, Phl p 7, are mainly of a conformational type because almost no IgE reactivity to unfolded synthetic peptides representing the N- as well as C-

terminal portion of Phl p 7 could be detected in the Phl p 7-reactive patients (Fig. 6a). It is also well established that IgE recognition of exogenous calcium-binding allergens (e.g., Phl p 7, grass pollen; Cyp c 1, carp parvalbumin) depends on protein-bound calcium (32–34). We therefore investigated the effects of calcium on IgE recognition of rHom s 4, rPhl p 7, and grass pollen allergens without calcium-binding domains (Phl p 2, Phl p 5) (Fig. 6b). IgE binding to rHom s 4 and rPhl p 7, but not to the other grass pollen allergens, was reduced or even abolished in the presence of the calcium-chelating agent EGTA (Fig. 6b).

Hom s 4 cross-reacts with calcium-binding exogenous allergens

The findings that Hom s 4-reactive patients were also sensitized to Phl p 7 and that Hom s 4 contains calcium-sensitive IgE epitopes prompted us to study whether IgE Abs from Hom s 4-reactive AD patients cross-react with calcium-binding exogenous allergens. Cross-wise IgE competition experiments were performed with the major fish allergen, carp parvalbumin, rCyp c 1 (34), and the grass pollen allergen, rPhl p 7 (Refs. 32 and 33 and Fig. 7) in a non-denaturing dot blot assay. Preincubation of Hom s 4-reactive sera with rHom s 4 yielded weak (patient 2) or no (patient 1) inhibition of IgE reactivity to the exogenous allergens (Cyp c 1, Phl p 7) (Fig. 7). Carp parvalbumin, rCyp c 1, completely inhibited (patient 2) or strongly reduced (patient 1) IgE recognition of Hom s 4 (Fig. 7).

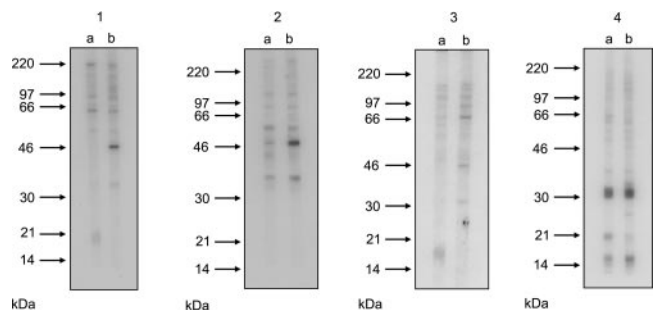


FIGURE 5. Subcellular distribution of rHom s 4. Nitrocellulose-blotted subcellular protein fractions (*panel 1*, mitochondria; *panel 2*, microsomes; *panel 3*, cytoplasm; *panel 4*, nuclei) were incubated with a rabbit anti-rHom s 4 antiserum (*lanes b*) or the rabbit preimmune serum (*lanes a*). Bound rabbit Abs were detected with ^{125} I-labeled donkey anti-rabbit Abs and visualized by autoradiography. Molecular masses (kilodaltons) are indicated on the left margins.

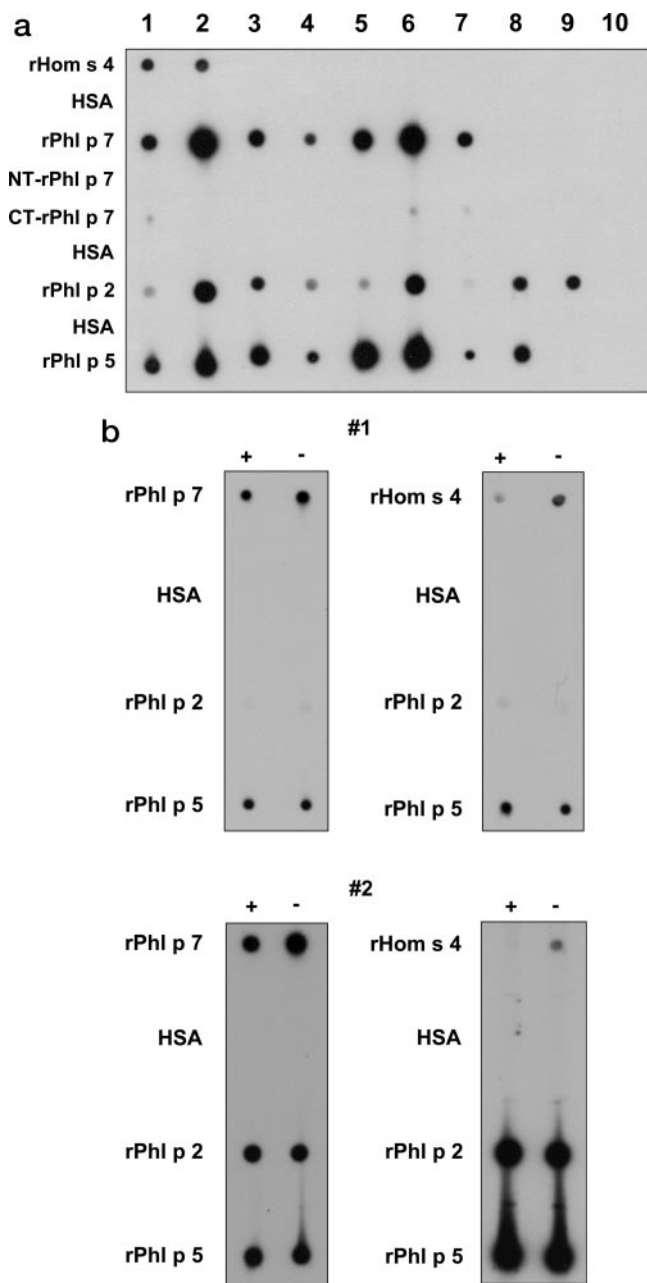


FIGURE 6. *a*, IgE recognition of rHom s 4. Serum samples from 10 allergic patients were exposed to nitrocellulose-dotted purified rHom s 4, recombinant grass pollen allergens (rPhl p 7, rPhl p 2, rPhl p 5), two peptides comprising the N-terminal (aa 2–37) or C-terminal (aa 36–78) portion of rPhl p 7, and HSA. *b*, Nitrocellulose-dotted recombinant grass pollen allergens (rPhl p 7, rPhl p 2, rPhl p 5), rHom s 4 as well as HSA were incubated with sera from two Hom s 4-reactive AD patients in the presence of EGTA (lane +) or CaCl_2 (lane –). Bound IgE Abs were detected with ^{125}I -labeled anti-human IgE Abs and visualized by autoradiography.

Complete inhibition of IgE binding to Hom s 4 was obtained with Phl p 7 (Fig. 7).

rPhl p 7 induces strong and rHom s 4 weak basophil histamine release

Next we compared rHom s 4 and rPhl p 7 regarding their capacity to induce basophil histamine release. Basophils from two AD patients containing Hom s 4- and rPhl p 7-specific IgE Abs were incubated with increasing allergen concentrations (Fig. 8, *a* and *b*).

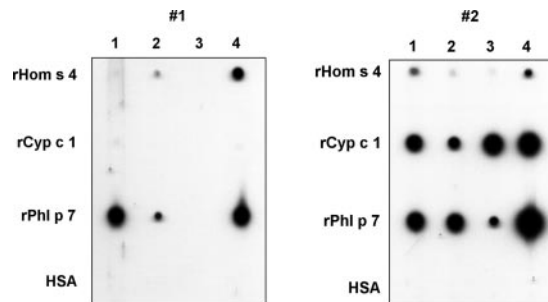


FIGURE 7. IgE cross-reactivity between rHom s 4 and exogenous calcium-binding allergens. Nitrocellulose-dotted rHom s 4, calcium-binding allergens from fish (carp: rCyp c 1), grass pollen (rPhl p 7) as well as HSA were exposed to sera from two rHom s 4-reactive AD patients (patients 1 and 2) which had been preadsorbed with rHom s 4 (lanes 1), rCyp c 1 (lanes 2), rPhl p 7 (lanes 3), or HSA (lanes 4). Bound IgE Abs were detected with ^{125}I -labeled anti-human IgE Abs and visualized by autoradiography.

In both patients, rPhl p 7 induced strong histamine release already at a concentration of 10^{-4} $\mu\text{g/ml}$. rHom s 4 did not induce significant histamine release up to a concentration of 10 $\mu\text{g/ml}$ in one patient (Fig. 8*a*) and was a weak inducer of histamine release in the other patient ($\sim 30\%$ histamine release at a concentration of 1 $\mu\text{g/ml}$; Fig. 8*b*). No induction of histamine release was found in an individual without Hom s 4-reactive IgE Abs up to 10 $\mu\text{g/ml}$ rHom s 4 (data not shown).

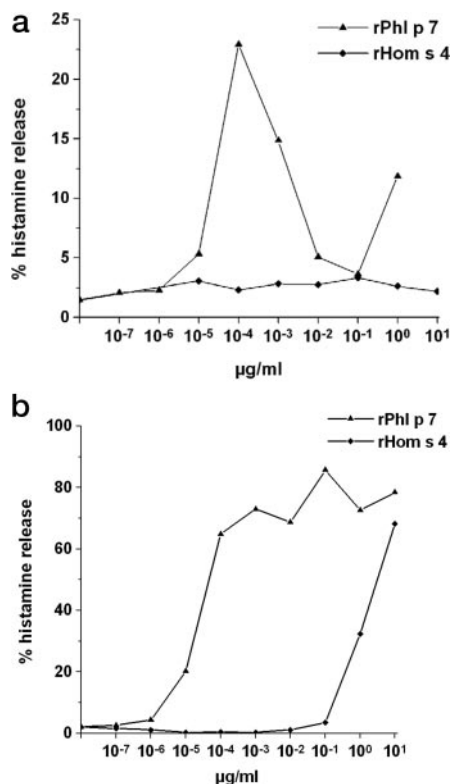


FIGURE 8. Induction of basophil histamine release. Basophils from two AD patients (*a* and *b*, respectively) containing rHom s 4-specific IgE Abs were incubated with increasing concentrations of rHom s 4 or the grass pollen allergen, rPhl p 7 (x-axis). The percentage of histamine release is displayed on the y-axis.

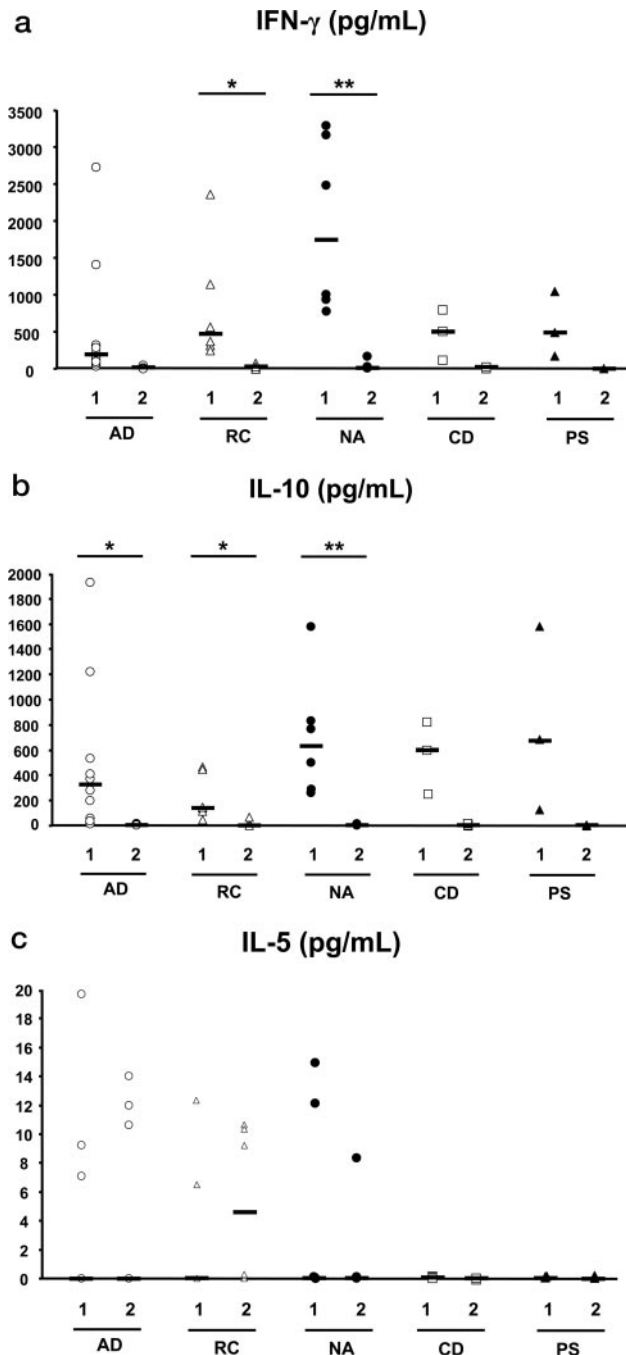


FIGURE 9. Cytokine responses. Hom s 4 (1)- and Phl p 1 (2)-specific cytokine levels (a, IFN- γ ; b, IL-10; c, IL-5) (picograms per milliliter) were determined in PBMC cultures from 28 individuals (AD, $n = 10$; RC, $n = 6$; NA, $n = 6$; CD, $n = 3$; PS, $n = 3$). Statistically significant differences are indicated (*, $p < 0.05$; **, $p < 0.01$).

rHom s 4 induces a Th1-biased immune response characterized by the release of IFN- γ

Next we investigated Hom s 4-specific cytokine and Ab responses in 28 individuals (AD, $n = 10$; RC, $n = 6$; NA individuals, $n = 6$; contact dermatitis (CD), $n = 3$; psoriasis (PS), $n = 3$; Table I). In PBMCs from each group of individuals, rHom s 4 induced stronger release of IFN- γ and IL-10 than the major timothy grass pollen allergen, rPhl p 1 (Fig. 9, a and b). No relevant differences regarding the induction of IL-5 were noted between Hom s 4 and Phl p 1 (Fig. 9c). Each of the tested individuals mounted IgG1

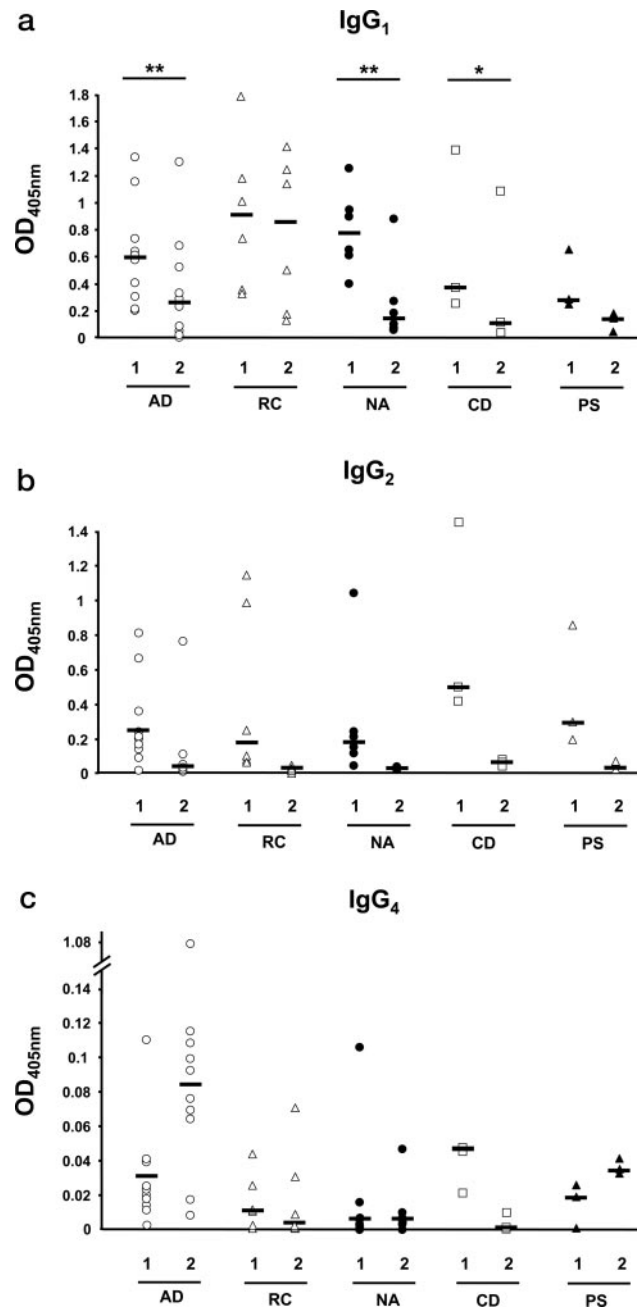


FIGURE 10. Ab responses. Hom s 4 (1)- and Phl p 1 (2)-specific Ab levels (a, IgG₁; b, IgG₂; c, IgG₄) were determined in sera from 28 individuals (AD, $n = 10$; RC, $n = 6$; NA, $n = 6$; CD, $n = 3$; PS, $n = 3$). Statistically significant differences are indicated (*, $p < 0.05$; **, $p < 0.01$).

responses to Hom s 4 that were higher than the Phl p 1-specific IgG1 levels in the AD, NA, CD, and PS groups (Fig. 10a). In agreement with the strong induction of IFN- γ in PBMC cultures, we found higher IgG₂ levels to Hom s 4 than to Phl p 1 in sera from each of the tested individuals (Fig. 10b). Hom s 4- and Phl p 1-specific IgG₄ Ab levels were generally low and heterogeneous in the different study groups (Fig. 10c).

The results of the cytokine and Ab determinations thus indicate that Hom s 4 has the intrinsic property to induce a Th1-biased immune response characterized by the induction of IFN- γ secretion and IgG₁ and IgG₂ Ab responses.

Table II. Cytokine responses in cord blood cultures^a

	IL-5 (pg/ml $\pm$ SD)	IL-10 (pg/ml $\pm$ SD)	IFN- γ (pg/ml $\pm$ SD)
CB ^a 1			
rHom s 4	7.0 $\pm$ 6.6	4.6 $\pm$ 1.3	2520 $\pm$ 1371.2
rPhl p 1	0	0	154.5 $\pm$ 19.7
TT	0	0	134.2 $\pm$ 21.7
IL-2	23.0 $\pm$ 10.5	148.3 $\pm$ 6.0	5114.7 $\pm$ 386.0
Medium	14.6 $\pm$ 10.3	0	254.2 $\pm$ 156.4
CB 2			
rHom s 4	15.9 $\pm$ 4.7	7.3 $\pm$ 1.2	688.6 $\pm$ 102.0
rPhl p 1	0	0	16.3 $\pm$ 9.8
TT	0	0	24.3 $\pm$ 17.5
IL-2	12.2 $\pm$ 11.5	418.7 $\pm$ 7.4	2850.0 $\pm$ 270.5
Medium	0	5.0 $\pm$ 1.6	27.4 $\pm$ 16.8
CB 3			
rHom s 4	0	12.1 $\pm$ 14.2	794.6 $\pm$ 86.0
rPhl p 1	0	0	8.3 $\pm$ 3.6
TT	0	0	0
IL-2	8.6 $\pm$ 10.6	226.1 $\pm$ 30.0	2113.4 $\pm$ 250.5
Medium	0	0	17.6 $\pm$ 13.0
CB 4			
rHom s 4	20.8 $\pm$ 10.3	10.0 $\pm$ 4.5	1163.6 $\pm$ 101.9
rPhl p 1	0	0	58.2 $\pm$ 29.5
TT	0	0	66.2 $\pm$ 31.0
IL-2	3.8 $\pm$ 0.5	388.2 $\pm$ 7.5	6706.5 $\pm$ 3120.7
Medium	0	0	29.9 $\pm$ 22.7
NA			
rHom s 4	0	687.2 $\pm$ 23.9	8689.8 $\pm$ 1763.8
rPhl p 1	12.4 $\pm$ 8.4	6.7 $\pm$ 2.9	69.3 $\pm$ 39.3
TT	59.4 $\pm$ 13.0	47.3 $\pm$ 10.7	8965.8 $\pm$ 3613.8
IL-2	59.1 $\pm$ 26.2	220.3 $\pm$ 52.4	9896.3 $\pm$ 969.7
Medium	10.1 $\pm$ 2.8	7.0 $\pm$ 6.0	61.1 $\pm$ 28.8

^a Cytokine responses (mean levels $\pm$ SD: IL-5, IL-10, IFN- γ) in response to rHom s 4, rPhl p 1, tetanus toxoid, IL-2, and medium alone are displayed for CBMC from four newborns (CB 1–4) and for PBMC from one NA adult.

Reactivity to Hom s 4 can be detected already in cord blood

To determine when reactivity to Hom s 4 is acquired, we compared cytokine production in response to Hom s 4 and two exogenous Ags (i.e., tetanus toxoid, grass pollen allergen Phl p 1) in CBMC cultures from four full term newborns and PBMC cultures from a NA adult (Table II). Similar to PBMC cultures, Hom s 4 induced strong release of IFN- γ in each of the four CBMC cultures (688.6–2520 pg/ml). Only PBMC from the adult individual mounted strong IFN- γ production to tetanus toxoid (8965.8 pg/ml), whereas no relevant IFN- γ response was found in any of the four CBMC cultures (Table II). The induction of IL-10 in CBMC cultures was much lower than that observed in PBMC cultures from adult individuals (Table II and Fig. 9b). The exogenous respiratory grass pollen allergen Phl p 1 did not induce relevant levels of IL-10, IFN- γ , or IL-5 in CBMC. No relevant IL-5 responses to the exogenous Ags (tetanus toxoid, Phl p 1) were noted in CBMC, and Hom s 4 induced varying low levels of IL-5. IL-2 induced the production of each cytokine (IL-5, IL-10, IFN- γ) in CBMC cultures (Table II).

Discussion

In patients suffering from severe and chronic manifestations of atopy (e.g., atopic dermatitis, chronic asthma) T cell-mediated immune reactions resembling features of delayed-type hypersensitivity predominate over immediate inflammation caused by IgE-mediated mast cell degranulation (7–9, 39). We have previously demonstrated that up to 60% of these patients exhibit IgE reactiv-

ity to autoantigens and isolated cDNAs coding for such IgE-reactive autoantigens (19–23). This study reports the recombinant expression, purification, and characterization of an IgE-defined autoantigen, Hom s 4. Hom s 4 belongs to a new subfamily of calcium-binding proteins and cross-reacted with exogenous calcium-binding allergens from plants and fish. IgE binding and competition experiments performed with Hom s 4 and exogenous calcium-binding allergens demonstrated that the exogenous calcium-binding allergens contained the majority of IgE epitopes. Absorption with Hom s 4 reduced IgE reactivity to Hom s 4 with little effect on reactivity to fish and plant allergens, whereas absorption with Phl p 7 or Cyp c 1 reduced IgE reactivity to Hom s 4, Phl p 7, and Cyp c 1. These findings indicate that Hom s 4-reactive patients were primarily sensitized to exogenous calcium-binding allergens and subsequently developed IgE cross-reactivity to self proteins, perhaps via molecular mimicry mechanisms. Comparable with previously described IgE-reactive autoantigens, Hom s 4 represented an intracellular protein (20, 22, 28, 30). It is predominantly expressed in the skin and may be released in the course of tissue injury occurring in the context of both atopic as well as nonatopic skin inflammation, scratching and superinfections.

Phl p 7, an exogenous calcium-binding allergen, induced strong histamine release from basophils of sensitized patients. By contrast, Hom s 4 triggered only weak IgE-dependent basophil degranulation which may be due to low Hom s 4-specific serum IgE levels or due to the fact that it does not cross-link receptor-bound IgE as effectively as Phl p 7. Nevertheless, it induced lymphoproliferative responses of magnitudes comparable with those induced by potent exogenous allergens. As has been reported for exogenous allergens (40, 41), Hom s 4-specific lymphoproliferative responses were not restricted to individuals containing Hom s 4-specific IgE Abs. In fact, we found that each of the tested individuals, including nonallergic individuals with no history of previous significant skin disease, mounted IgG₁ and IgG₂ Ab responses to Hom s 4 which were stronger than those against the exogenous major pollen allergen, Phl p 1. Furthermore, Hom s 4 induced stronger IFN- γ responses than Phl p 1 in PBMC cultures from each of the tested individuals. No relevant differences were found regarding IL-5 and IgG4 responses, supporting the assumption that Hom s 4 is an Ag that through intrinsic properties induces a Th1-biased immune response. Hom s 4-induced production of IFN- γ but not of IL-10 was detectable in CBMC cultures, suggesting that T cell reactivity to Hom s 4 is already acquired prenatally. Similar results were obtained for environmental allergens to which prenatal exposure is possible (42).

Because all recombinant allergens used in our experiments were obtained by expression in *E. coli* using similar strains and vectors and contained comparably low levels of endotoxin, our findings thus suggest that intrinsic features of Ags may influence the quality of the resulting immune response. Especially interesting was the finding that an IgE-defined autoantigen such as Hom s 4 can induce strong Th1 immune responses not only in individuals without a Th2-biased immune response (e.g., nonatopic persons, patients suffering from nonatopic chronic skin diseases) but also in Th2-prone severely atopic patients. In this context, it is noteworthy that two other IgE-defined autoantigens, Hom s 1 and Hom s 3 (cytokeratin II) have been described as potent targets for CTLs (20, 43–45). Furthermore, we found that another IgE-reactive autoantigen, Hom s 2, is also a potent inducer of lymphoproliferative responses and release of IFN- γ (Ref. 30 and I. Mittermann and R. Valenta, unpublished observations). Regarding AD, release of intracellular IFN- γ -inducing autoantigens in the course of tissue damage may explain the frequently observed occurrence of Th1-dominated infiltrates in sites of chronic atopic inflammation (11,

46, 47). However, it is equally possible that IFN- γ -inducing autoantigens such as Hom s 4 may contribute also to a variety of other nonatopic chronic inflammatory processes in the skin. Should further studies provide evidence that autoantigen-induced Th1 responses contribute both to chronic atopic as well as NA skin inflammation, it may be considered to develop therapeutic strategies which aim to down-regulate reactivity to these autoantigens.

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Disclosures

The authors have no financial conflict of interest.

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