Supplementary Material 1; Methods

Methods
Generation of monoclonal antibodies to the Can f 4 allergen

The skin prick test extract of dog epithelium was purchased from ALK Abelló (Hørsholm, Denmark). The concentrated extract was subjected to gel filtration on the Superdex 75 preparative grade column XK 16/70 (GE Healthcare Bioscience AB, Uppsala, Sweden) with the GE Healthcare Äktapurifier HPLC system (GE Healthcare Bioscience AB). PBS was used as the eluent with a flow rate of 1 mL/min. Seventy fractions of 1 ml were collected and examined by sodium dodecylsulphatepolycrylamide gel electrophoresis (SDS-PAGE), as described previously. The fractions containing the proteins of interest (12-18 kDa) were pooled and concentrated with the Microsfe 3K devise (Pall Gelman Laboratories, MI, USA). BALB/c mice were immunized intraperitoneally with 50 μg of the antigen combined with the Freund’s complete adjuvant, basically as described previously. Three subsequent immunizations were done at four-week intervals with 25 μg of the antigen together with the Freund’s incomplete adjuvant. The mice were sacrificed four days after the last immunization and the spleen cells were fused with the Ag-8 mouse myeloma cell line using hybridoma fusion and cloning supplement HFCS (Roche, Penzberg, Germany), according to the manufacturer’s instructions. Experiments were performed in agreement with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg March 18, 1986, adopted in Finland May 31, 1990). The study was approved by the Animal Care and Use Committee of the University of Kuopio (now the University of Eastern Finland) with the permit number 04-37/2004.

Hybridoma supernatants were tested for reactivity to the 18 kDa component of dog dander with ELISA and immunoblotting. The hybridomas secreting specific antibody to the 18 kDa component were cloned two to three times by the limiting dilution method and were then further propagated. The antibodies 26D, 41G and 48F were purified by affinity chromatography on a protein-G column (GE Healthcare, USA). The isotypes of the antibodies were tested by the Clonacell InstantCHEK Oneminute isotype Kit (StreamCell, Vancouver, Canada). Protein concentrations were determined by the Bio-Rad Protein Assay Kit using bovine gamma globulin as a standard. Epitope specificity of the mAbs obtained was assessed, as described below.

Biochemical characterization of nCan f 4

For molecular weight and purity determination, the affinity-purified Can f 4 was analyzed by SDS-PAGE and silver staining. For characterizing the internal disulfide bridges, SDS-PAGE was performed with various amounts of dithiothreitol in the sample buffer (0.006-0.78 mM). The amount of the allergen was 0.3 μg/lane.

For the determination of molecular mass by MALDI-TOF mass spectrometry, the affinity-purified allergen was further subjected to RP-HPLC in a TSK TMS 250 column. The eluted protein was subjected to mass spectrometric analysis using an Ultraflex TOF/TOF instrument (Bruker Daltonics, Bremen, Germany). For the N-terminal sequence analysis, RP-purified protein was subjected to SDS-PAGE and electroblotting to a PVDF membrane. The 18 kDa protein band was then subjected to Edman degradation using a Procise 494 A sequencer (Applied Biosystems, Perkin Elmer, CA, USA). For digestion with trypsin, the RP-HPLC-purified allergen was reduced with diithiothreitol, alkylated with 4-vinylpyridine and digested with trypsin. The resulting peptides were separated with RP-HPLC using a C18-column (1 mm × 150 mm, 300 Å, 5 μm) with a linear gradient of acetonitrile (0-40% over 120 min) at a flow rate of 50 μL/min, and A214 was monitored. The tryptic peptides were then subjected to Edman degradation.

Construction of the gene encoding Can f 4

In brief, the database of the Canis lupus familiaris genome from the National Center for Biotechnology (Washington, D.C.; http://www.ncbi.nlm.nih.gov/BLAST) was screened with the peptide sequences obtained by Edman degradation of the nCan f 4 allergen, using a protein query to search the translated nucleotide database for homology. The analysis was complemented by comparing the sequential information of known odorant-binding proteins (OBP; Sus scrofa OBP, UniProtKB, P81245 and Bovis taurus OBP, UniProtKB, P07435) with the sequential information of the nCan f 4 peptides obtained by the Edman degradation. The genes encoding OBPs have a leader sequence and this was also taken into account when the screening was conducted. The specific primers for the Can f 4 cDNA amplification were designed according to the screening result (see Fig. E2 and Table E1).

Total RNA was isolated from the mixture of tongue, mandibular and parotid gland tissue samples of a single dog (Beagle, male) by the RNAgents® total RNA isolation system (Promega, Madison, WI, USA) following the manufacturer’s instructions. The total RNA obtained was utilized for cDNA synthesis using oligo(dT)16 primers and AccuScript reverse transcriptase (Stratagene, LA Jolla, CA, USA) according to the supplier’s instructions. The oligo(dT)16 primed cDNAs were used with gene specific primers to amplify the fragments of the dog allergen using Phusion DNA polymerase (Finnzymes Oy, Espoo, Finland). PCR cycle conditions were the following: initial denaturation (98°C, 3 min), followed by 35 cycles of amplification (98°C, 15 s), annealing (57°C, 30 s), extension (72°C, 40 s) and final extension (72°C, 7 min).
To subclone and sequence the amplicons, right-size products were gel-extracted using the Ultrafree-DA Centrifugal Filter Device (Millipore, Bedford, MA, USA). Then, Biotools DNA polymerase (Biotools, Madrid, Spain) was used to add 3’ A overhang to purified PCR fragments for UA-cloning by the Qiagen PCR cloning plus kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The sequencing was purchased from the AIV Institute, Kuopio, Finland. The theoretical MW and isoelectric point of Can f 4 were determined using the ExpPASy Prot Param tool (http://au.expasy.org/tools/pi_tool.html). Prediction of the signal peptide was made using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/).

The GenBank accession number for the sequence of full-length cDNA of the Can f 4 variant reported is KF 192077.

Production and purification of recombinant Can f 4
To generate the DNA fragment encoding the Can f 4 sequence for cloning into the yeast, *Pichia pastoris* pPIC9 expression plasmid-specific primers were designed based on the settled nucleotide sequence. In addition, a polyhistidine tag sequence with Factor Xa restriction site was included in the 5’ primer. The achieved construct was cloned into the pPIC9 expression vector. The nucleotide sequence of the fragment was confirmed by DNA sequencing using the Thermo Sequenace CY5 Dye Terminator Kit and the A.L.F Express DNA Sequencer (GE Healthare Bioscience AB). *P. pastoris* yeast was transformed with the construct pPIC9rCan f 4 according to the manufacturer’s instructions (Invitrogen, San Diego, CA, USA).

The allergen was produced according to the instructions of the manufacturer of the *P. pastoris* expression system (Invitrogen, San Diego, CA, USA). The culture supernatant was harvested by centrifugation and the histidine-tagged rCan f 4 protein was purified with the HisTrap Kit (GE Healthare Bioscience) according to the manufacturer’s instructions. However, rCan f 4 was mostly affinity-purified as nCan f 4 using the mAbs, as described in Methods. The subsequent gel filtration of the recombinant protein was performed with the Superdex 75 preparative grade media column on the GE Healthcare Äktapurifier HPLC system with PBS as the eluant. Calibration curve for the column was obtained using the molecular-weight standard kit, MWGF70 (Sigma-Aldrich, St. Louis, USA). The monomeric and multimeric rCan f 4-containing fractions were separately collected and pooled. The purity of the allergen preparations was verified by SDS-PAGE gel electrophoresis and silver staining.

For the determination of molecular mass of rCan f 4, all spectra were obtained by ESI-MS using the QSTAR XL Quadrupole-TOF mass spectrometer (Applied Biosystems) with MDS Sciex Nanospray. Sample solutions were loaded in Proxeon emitters and subjected to electrospray-MS. Mass range was m/z 600-5,000. For deconvolution of the multiply charged in the spectra, the Bayesian Protein Reconstruct algorithm of Analyst Qs1.1 software (Applied Biosystems) was used. Protein concentrations were determined by absorbance spectroscopy using the molar absorption coefficients (http://us.expasy.org/tools/protparam.html). Sterile-filtered preparations were stored at -70°C.

Dot blot
The reactivity of human IgE and the mAbs against the denatured or nondenatured nCan f 4 was analysed with the dot blot method. In brief, 300 ng of the nondenatured allergen or the allergen denatured with 4% SDS and 5% 2-mercaptoethanol were dotted onto a nitrocellulose membrane using the HybriDot Manifold device (BRL, Gaitherburg, USA). Then, the membrane was incubated overnight with sera or the mAbs labeled with biotin (See below). After an incubation with HRP-labeled mouse anti-human IgE (1:2,000, 2 h, valmistaja) or strepavidin-HRP conjugate (1:30,000, 0.5 h; GE Healthcare, Buckinghamshire, England), respectively, the dots were visualized with the ECL detection reagents (GE Healthcare, Buckinghamshire, England). The dot intensities were measured with the ImageJ software version 1.4 (National Institute of Health, Bethesda, USA; http://rsb.info.nih.gov/ij/) using the plugin Microarray Profile.jar algorithm (http://www.optinav.com/download/MicroArray_Profile.jar).

References