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The NPC2 protein A novel dog allergen

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ABSTRACT

Background: Dogs are an important source of indoor allergens that cause rhinoconjunctivitis, urticaria, and asthma in sensitized individuals. Can f 1 is reported as a major dog allergen, but other allergens have also been identified. Identification of immunologically important allergens is important for both the diagnosis and treatment of dog allergy.

Objective: To identify and characterize the canine NPC2 protein, a novel dog allergen.

Methods: We screened commercial and laboratory-generated aqueous dog extracts by 2-dimensional polyacrylamide gel electrophoresis with IgE immunoblotting using human serum samples from 71 dog-allergic individuals. A target of interest was excised from the gel and sequenced. Canine NPC2 sequence was generated, and recombinant proteins expressed in yeast and bacteria were used to determine allergenicity. An IgE enzyme-linked immunosorbent assay was used for screening 71 dog-positive and 30 dog-negative serum samples.

Results: A 16-kDa protein ($pK = 8.5$) in dog allergen extracts was recognized by specific IgE. The protein was identified by sequencing as a CE1 protein or NPC2 protein. Human IgE bound to recombinant protein was expressed in both yeast and bacteria. Ten (14%) of 71 individuals had specific IgE to NPC2 protein from bacteria, and 12 (17%) had IgE to NPC2 protein from yeast. Binding of pooled dog-allergic serum IgE to the dust mite protein Der p 2 was partially inhibited by recombinant NPC2 protein.

Conclusions: NPC2 protein, a member of the MD-2–related lipid recognition family, is identified as a dog allergen (Can f 7), with an apparent seroprevalence of 10% to 20%.

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Introduction

Exposure to dog allergens induces rhinoconjunctivitis, urticaria, and asthma in sensitized individuals, affecting 5% to 10% of the adult population worldwide.^{1–4} Six dog allergens have been identified (www.allergen.org). Can f 1, a salivary lipocalin, is recognized as a major dog allergen, and other known allergens include albumin (Can f 3), the prostatic kallikrein Can f 5, and the lipocalins Can f 2, Can f 4, and Can f 6. The lipocalins have apparent molecular masses of 19 to 29 kDa.^{5–10}

Commercial dog allergen extracts are obtained from dog hair, dander, and epithelium.^{2,11,12} Recent studies have highlighted the problem of false-negative skin test results with some dog allergen extracts,^{2,11} drawing attention to the choice of source materials and extraction conditions as important factors in determining product quality. Recently, 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled to mass spectrometry (MS) was used to

identify 4 novel human IgE-binding proteins from dog saliva: ANGPTL5, BPIFA2, mucin-5B, and IgA heavy chain constant region.²

In this study, we identify another dog allergen, NPC2 protein, also known as CE1 protein. Dog NPC2 protein is a 16-kDa allergen in the MD-2–related lipid recognition (ML) family. The World Health Organization/International Union of Immunological Societies designation is Can f 7 (<http://allergen.org/viewallergen.php?aid=850>).

Methods

Preparation of Dog Epithelium and Dog Hair Extracts

Dog allergen extracts were prepared in house from defatted dog hair and dog epithelium source materials (Greer Laboratories, Lenoir, North Carolina), which were mixed with phosphate-buffered saline (PBS) that contained protease inhibitor cocktail (Sigma-Aldrich, St Louis, Missouri) overnight at 4°C, separated by centrifugation at 10,000g for 30 minutes at 4°C, and further clarified by sequential filtration (0.80 and 0.45 μ m). The clarified dog allergen extracts, manufactured in the Laboratory of Immunobiochemistry from dog hair or dog epithelium powder (and designated as LIB-hair and LIB-epi, respectively), were aliquoted in glass vials and stored

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at -20°C . Additional dog extracts were purchased from US allergen extract manufacturers (eTable 1). Total protein content was estimated using bicinchoninic acid assay with bovine albumin as the standard (Thermo Fisher Scientific Inc, Norcross, Georgia).

SDS-PAGE

One-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed to analyze the proteins in the extracts under reducing conditions. Equal amounts of extracts were mixed with sample buffer and heated at 95°C for 10 minutes before separation on 4% to 12% Bis Tris gels. The bands were visualized with silver nitrate (Life Technologies, Carlsbad, California).

2D-PAGE

The extracts were clarified using 2D Cleanup Kit (BioRad Laboratories, Hercules, California) and rehydrated with denaturing and solubilizing buffer (8 M urea, 3.22%[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 0.806% ampholytes, 3.2% bromophenol blue, 100 mM dithiothreitol). The protein samples were focused on pH 3 to 10 nonlinear ZOOM strips (Life Technologies) followed by focusing at 500 V for 3 hours. The focused proteins were reduced for 15 minutes followed by alkylation with 125 mM iodoacetamide for 15 minutes at room temperature. Reduced and alkylated strips were loaded on 4% to 20% Tris-glycine gels for separation. The separated protein spots were fixed (40% methanol and 10% acetic acid) and stained (Simply Blue Safe Stain; Life Technologies). The spots of interest were manually excised, destained, and digested with trypsin for liquid chromatography–tandem mass spectrometry analysis (Applied Biomics, Hayward, California). The Mascot search engine (Applied Biomics) was used for identification of proteins from the sequenced peptides.

Human Serum IgE Immunoblotting

Dog-allergic human serum samples that tested positive for dog specific IgE were purchased from Plasma Labs International (Everett, Washington). For screening of dog-allergic human serum samples, the extracts or recombinant proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (pore size, 0.2 μm ; Life Technologies), which was blocked with Starting Block (Life Technologies) and incubated with individual or pooled dog-allergic human serum samples (1:10). Binding of human serum IgE was detected by biotinylated goat antihuman IgE (1:2500 diluted in starting block) followed by streptavidin-horseradish peroxidase (HRP) (1:10,000 diluted in PBS). The recombinant NPC2 proteins (bacterial and yeast) were also detected using HRP anti-myc clone 9E10 (1:1000; Thermo Fisher Scientific) or HRP anti-6 $\times$ His antibodies (1:1000; Life Technologies). The signals were visualized by West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

Expression and Purification of Recombinant Bacterial and Yeast NPC2 Proteins

Canine NPC2 complementary DNA was synthesized (GenScript, Piscataway, New Jersey) and cloned at *EcoRI* and *NotI* sites into the bacterial expression plasmid pET-24a(+) (Novagen, Danvers, Massachusetts). BL21DE3 (Life Technologies) competent cells were transformed with pET24a-CfNPC2 plasmid, and transformants were selected in the presence of kanamycin (30 $\mu\text{g}/\text{mL}$). For expression of recombinant bacterial NPC2 (rB-NPC2), the culture, grown to an OD_{600} of 0.6 to 0.8, was induced with 100 μM isopropyl-1-thio- β -galactopyranoside. After 5 hours of induction, cells were pelleted, and rB-NPC2 protein was purified from the soluble fractions using nickel ion affinity chromatography (His-select

Nickel; Sigma Life Science, St Louis, Missouri) as previously described.¹³ The protein content of the purified fractions was estimated using bicinchoninic acid assay and analyzed by SDS-PAGE.

For expression of recombinant yeast NPC2 (rY-NPC2), NPC2 complementary DNA was cloned in pPICZB plasmid (Life Technologies) at *EcoRI* and *NotI* sites under the control of the AOX promoter. The plasmid has Myc and His tags at the 3' end for purification and detection. The insertion of the coding sequence was verified by sequencing (Center for Biologics Evaluation and Research Core Facility, US Food and Drug Administration). *Pichia pastoris* X-33 cells (Life Technologies) were transformed with linearized and purified pPICZB-CfNPC2 plasmid by electroporation using Gene Pulser ECM630BTX (Harvard Apparatus, Holliston, Massachusetts), pulsing at 1500 V, 200 Ohms, 50 μF , and 6 to 8 milliseconds. The transformants were selected on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol) plates that contained 100 $\mu\text{g}/\text{mL}$ of Zeocin. For expression, overnight grown culture was resuspended in buffered glycerol complex medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate [pH 6.0], 1.34% yeast nitrogen base, 1% glycerol) and incubated at 30°C for an additional 8 hours until the OD_{600} reached 2.0. Cell pellets were resuspended in buffered methanol complex medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate [pH 6.0], 1.34% yeast nitrogen base, 0.5% methanol) to an OD_{600} of 1.0 and incubated at 30°C for 3 additional days, replenishing 0.5% methanol every 24 hours to maintain induction. After induction, culture was pelleted by centrifugation and used immediately for purification or stored at -20°C until use.

The cell paste was lysed in ice-cold buffer (pH 8.0) that contained 50 mM sodium phosphate, 5% glycerol, 10 mM imidazole, and protease inhibitor cocktail. The soluble fraction was collected and incubated with Talon metal affinity resin (Clontech, Mountain View, California) with end-over-end mixing in a gravity column followed by washing with 50 mM sodium phosphate buffer (pH 8.0) and 20 mM imidazole. The rY-NPC2 was eluted with 50 mM sodium phosphate buffer (pH 8.0) that contained 250 mM imidazole. The purified protein fractions were analyzed by SDS-PAGE and immunoblotting using anti-myc and anti-His antibodies.

PNGase F Treatment

rY-NPC2 (1.0 μg) was treated with 250 U of PNGase F (New England BioLabs Inc, Ipswich, Massachusetts) at 37°C for 1 hour. The deglycosylated protein was tested for mobility shift by SDS-PAGE under reducing conditions. RNase B (Sigma-Aldrich, St Louis, Missouri) was used as positive control.

Seroprevalence

House dust mite serum pool S8Dpf was obtained from the Center for Biologics Evaluation and Research, US Food and Drug Administration.¹⁴ Sera collected from dog-allergic individuals and house dust mite human serum pool S8Dpf were tested for IgE binding to rB-NPC2 and rY-NPC2. Microtiter plates (Nunc, Maxisorp, Thermo Fisher Scientific, Rochester, New York) were coated with rY-NPC2 and rB-NPC2 (10 $\mu\text{g}/\text{mL}$). After blocking, coated wells were exposed sequentially to diluted serum samples (1:10), biotinylated goat antihuman IgE antibody (1:2000) (KPL, Gaithersburg, Maryland), streptavidin-HRP (1:10,000), and tetramethylbenzidine substrate solution (KPL). Reaction was stopped by adding 0.5 M sulfuric acid (100 mL per well), and absorbance was measured at 450 nM. Sera from dog nonallergic individuals were used as negative control. Each human serum sample was tested 3 times against rY-NPC2 and rB-NPC2 with nearly identical results; a representative experiment is reported. Sera yielding enzyme-linked immunosorbent assay absorbances higher than the 97th quantile for dog nonallergic individuals were considered positive to dog NPC2.

Inhibition Enzyme-Linked Immunosorbent Assay

Microtiter plate wells were coated overnight at 4°C with *Der-matophagoides pteronyssinus* extract (1:50) (Antigen Laboratories, Liberty, Missouri), nDer p 1 (25 µg/mL), and nDer p 2 (25 µg/mL) (Indoor Biotechnologies, Charlottesville, Virginia). Diluted S8Dpf human serum pool was mixed with increasing concentrations of rY-NPC2 or rB-NPC2. After washing, the wells were blocked, human serum that contained rY-NPC2 or rB-NPC2 was added to the duplicate wells, and the plate was incubated for 3 hours at room temperature. Biotinylated goat antihuman IgE (1:2500) (KPL), streptavidin-HRP (1:2500), and tetramethylbenzidine substrate solution, in sequence, were used to detect binding of IgE. Assays were repeated 2 times, and the mean percentage inhibition values are reported. Percentage inhibition was calculated as $100 \times [(OD(\text{no inhibitor}) - OD(\text{inhibitor})) / OD(\text{no inhibitor})]$.

Results

Selection of Dog Allergen Extract

LIB-hair extract, LIB-epi extract, and dog allergen extracts from 6 US manufacturers had large compositional heterogeneity on SDS-PAGE (Fig 1A). Extracts from 3 manufacturers had only a band at 70 kDa, whereas extracts from 2 other manufacturers had several bands at 17 to 150 kDa. No protein bands were detected in the extract obtained from 1 other manufacturer. LIB-epi and LIB-hair extracts had numerous discrete protein bands higher than 76 kDa. Extracts from 3 manufacturers were selected for further analysis.

Dog-Allergic Human Serum Pool

Human serum samples from 71 dog-allergic individuals had dog specific IgE levels of 20 to 100 kU/L or more and total IgE from 140

to 1677 kU/L (data not shown). The serum samples were screened against LIB-epi (eFig 1A) and LIB-hair (eFig 1B) extracts. Eighteen serum samples recognized a protein band migrating at approximately 70 kDa in LIB-epi (eFig 1A); 25 serum samples recognized a 27-kDa protein band in LIB-hair (eFig 1B). Serum samples of 20 individuals did not recognize any of the proteins in LIB-epi or LIB-hair extracts. The pool was prepared by combining equal parts of 51 serum samples that recognized proteins in both epithelia and hair extracts.

The serum pool was tested by IgE immunoblot against LIB-epi, LIB-hair, and 3 commercial extracts; nCan f 1 was used as a control (Fig 1B). IgE in the serum pool bound to nCan f 1; to the band at 25 kDa in 1 manufacturer extract and, to a lesser degree, LIB-hair; and to bands at 70 kDa in LIB-hair, LIB-epi, and all 3 commercial extracts. Binding of serum pool IgE to other bands was noted in LIB-epi (>100 kDa) and 2 manufacturer extracts (>100, 31, and 40 kDa).

Identification and Expression of Dog NPC2

Binding of serum pool IgE to proteins in dog extracts was further analyzed by 2D-PAGE. A Coomassie-stained 2D-PAGE gel of the LIB-epi extract revealed multiple protein spots (Fig 2A), and human serum pool recognized a 70-kDa protein band in the immunoblot (Fig 2B). In addition a 17- to 20-kDa protein band with pH of approximately 8.5 was also recognized (Fig 2B).

A Coomassie-stained 2D-PAGE gel of the extract from manufacturer A revealed a 70-kDa band and multiple protein spots between 17 and 20 kDa (Fig 2C). Serum IgE immunoblotting of replicate gel (Fig 2D) had clear signals at 17 and 20 kDa. Like LIB-epi extract, a distinct spot at approximately 16 kDa and high pH was also observed in 1 manufacturer extract. This protein spot was excised from the replicate Coomassie-stained gels and sequenced for peptide mapping.

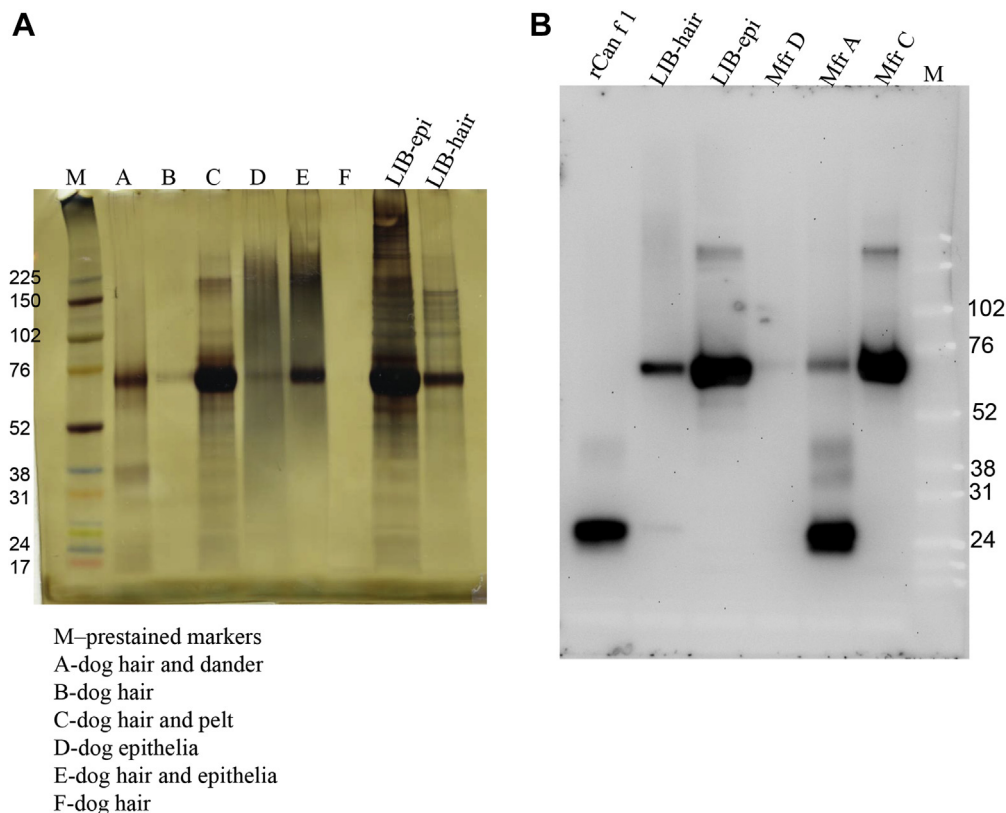


Figure 1. A, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of dog allergen extracts. B, Immunoblot of dog allergen extracts and nCan f 1, with dog-allergic human serum pool (1:10) and biotinylated antihuman IgE (1:2500).

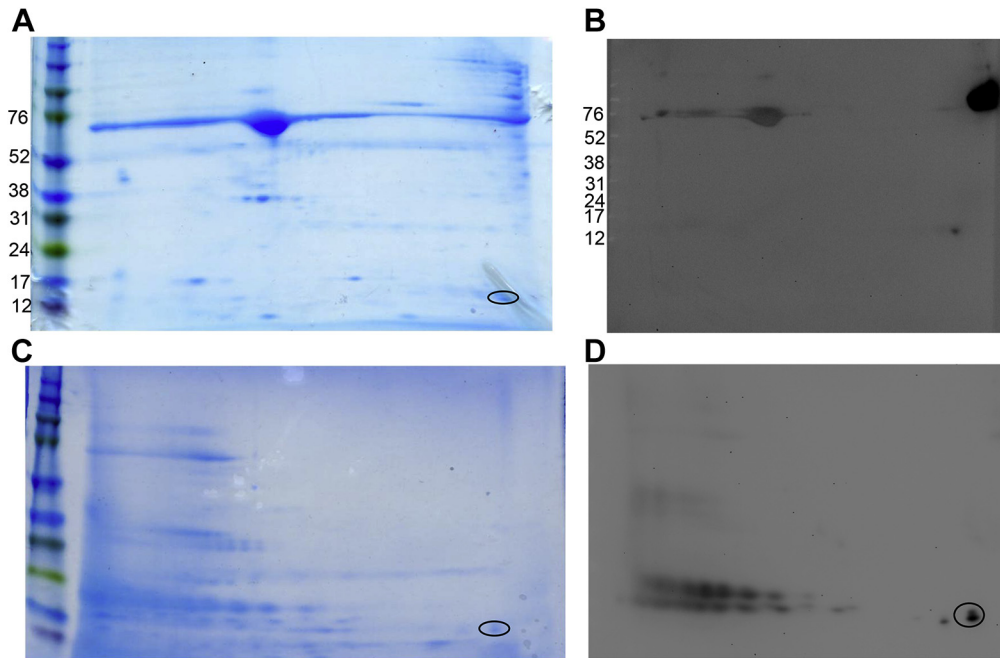


Figure 2. Two-dimensional polyacrylamide gel electrophoresis of Laboratory of Immunobiochemistry dog epithelial allergen extract (LIB-epi) (A) and commercial dog hair and pelt (C). Immunoblots of LIB-epi (B) and commercial dog hair and pelt (D). The blots were developed as in Figure 1B. The excised protein spot is indicated (oval).

Matrix-assisted laser desorption ionization time of flight sequencing yielded 6 unique peptides sequences (Fig 3A) of a 16-kDa molecular weight protein (Table 1). Basic Local Alignment Search Tool analysis of these peptides had significant matches with canine NPC2 protein (GenBank accession NP_001003242.2).

Dog NPC2 protein is a 149–amino acid protein with a 21–amino acid N–terminal signal peptide. Like other mammalian NPC2

proteins, it has 6 conserved cysteine residues and 3 potential glycosylation sites: NVN, NVT, and NQH (Fig 3A). Dog NPC2 protein belongs to the ML domain. ML domain is present in proteins from plants, animal, and fungi.¹⁵ Dog NPC2 shares 75% identity with human NPC2. The group 2 allergens of house dust mite, Der p 2 and Der f 2, also belong to this family and share 23% identity with dog NPC2 (Fig 3B).

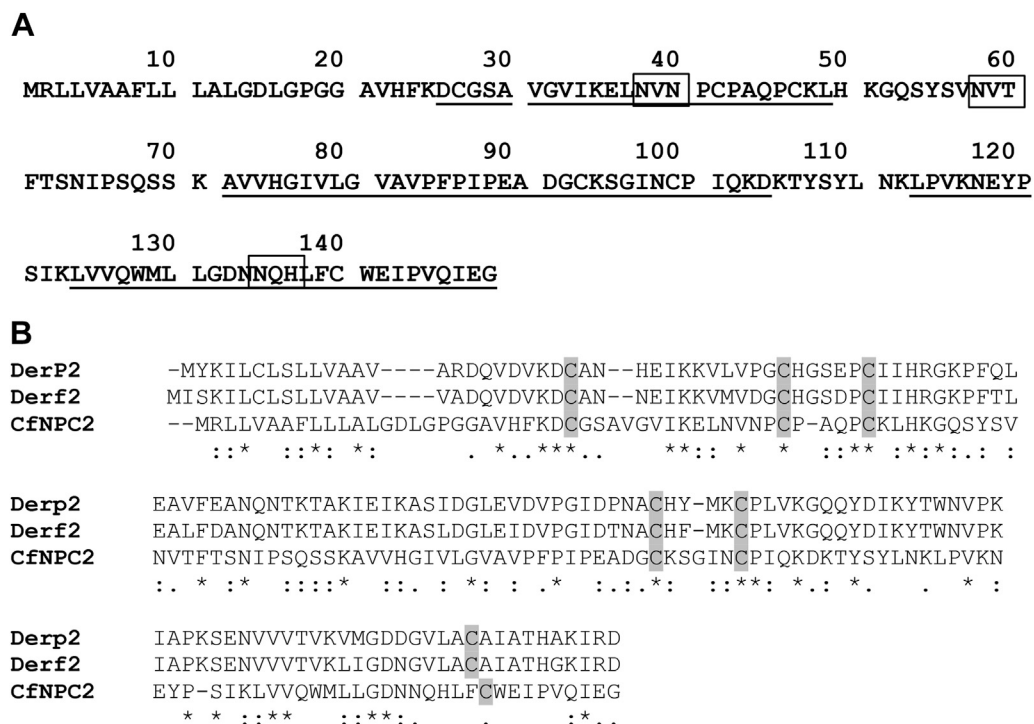


Figure 3. A, Full-length sequence of dog NPC2. Unique peptides sequences are underlined. Conserved cysteine residues and 3 potential glycosylation sites are boxed. See text for discussion. B, Sequence alignment of dog NPC2 and group 2 dust mite allergens. Conserved cysteine residues are shaded. Asterisks indicate the group 2 allergens of house dust mite, Der p 2 and Der f 2, also belong to this family and share 23% identity with dog NPC2. See text for discussion.

Table 1
Peptide Sequences of Dog NPC2 Protein*

Calculated Mass (Da)	Observed Mass (Da)	Start Sequence	End Sequence	Sequence
1005.5032	1005.3569	26	35	DCGSAVGVK
1016.5193	1016.3625	95	103	SGINCPQK
1287.7307	1287.5515	113	123	LPVKNEYPSIK
1526.709	1526.4725	36	48	ELNVNPCPAQPK
2345.2686	2344.8821	72	94	AVVHGIVLGVAVPFPIPEADGCK
2345.2686	2344.8821	72	94	AVVHGIVLGVAVPFPIPEADGCK
3154.5488	3154.0154	124	149	LVVQWMLLGDNNQHLFCWEIPVQIEG

*For the dog NPC2 protein (accession No. NPC2_CANFA), protein molecular weight was 16045.3 Da, protein isoelectric point was 8.21, and peptide count was 6.

Dog NPC2 was expressed in *Pichia pastoris* (rY-NPC2) and in *Escherichia coli* (rB-NPC2). Recombinant protein was purified from the soluble fractions of yeast and bacterial cell lysates.

Expression of rY-NPC2 was confirmed by immunoblotting cell lysates at various time points during induction using anti-myc antibodies; maximum expression was observed at 48 hours (data not shown). SDS-PAGE analyses of multiple eluates of recombinant proteins revealed highly pure rY-NPC2 (24 kDa) and rB-NPC2 (20 kDa) (data not shown). The recombinant proteins were localized with antihistidine antibodies (Fig 4A) and confirmed by mass spectrometric sequencing (data not shown). IgE antibodies in the dog-allergic human serum pool recognized both rY-NPC2 and rB-NPC2 (Fig 4B).

Immunological Reactivity of rNPC2 Proteins

Serum IgE prevalence was estimated against rY-NPC2 and rB-NPC2 proteins. The proteins were immobilized separately on microtiter plates at 10 µg/mL, and IgE binding was assessed using

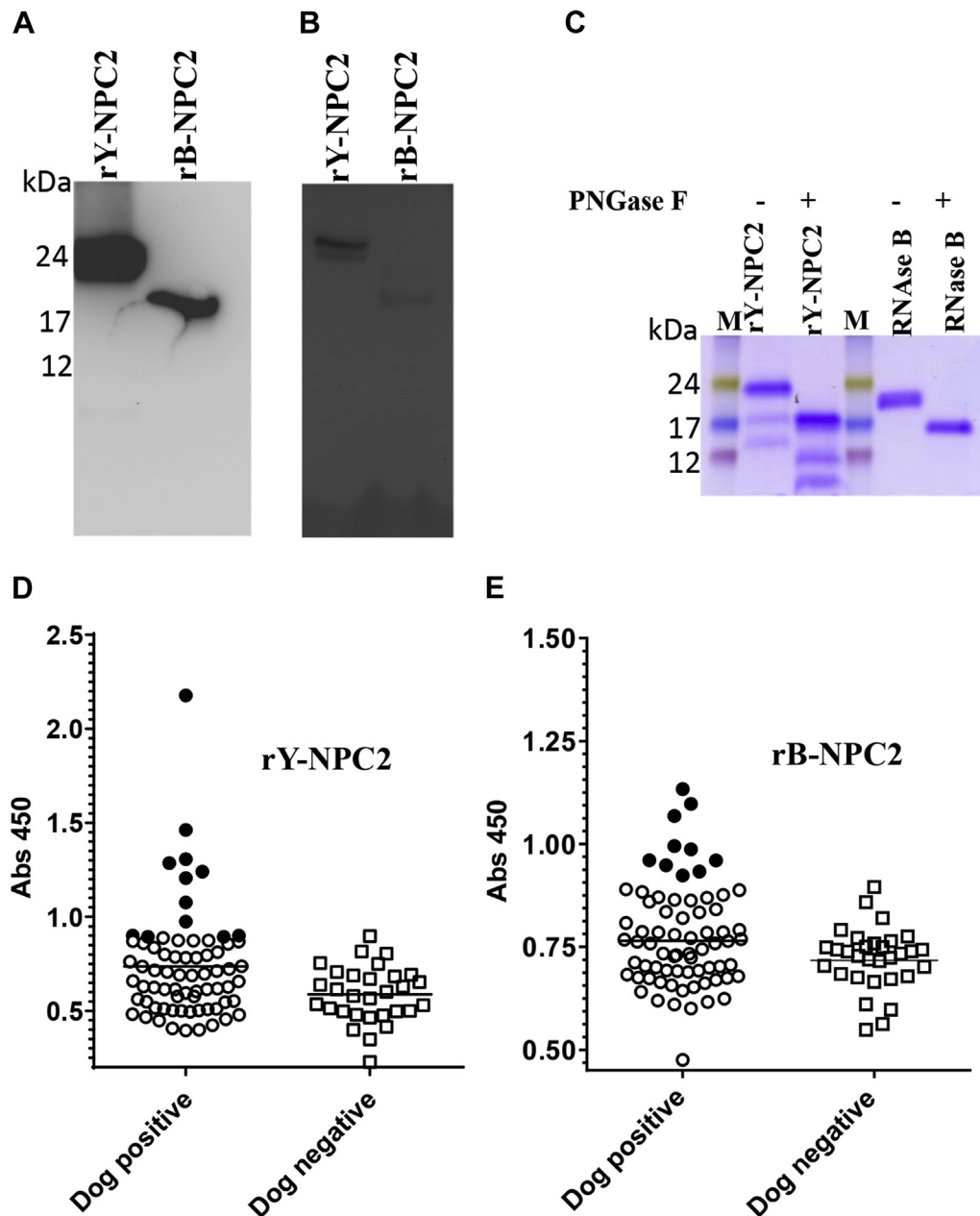


Figure 4. Immunoblot of recombinant yeast NPC2 (rY-NPC2) and recombinant bacterial NPC2 (rB-NPC2) proteins using anti-His antibody (A) and dog-positive human serum pool (B). PNGase F treatment of rY-NPC2 (C). IgE seroprevalence of rY-NPC2 (D) and rB-NPC2 (E). Horizontal lines represent geometric means. Filled circles represent values in the greater than 97th quantile (see text).

serum samples from 71 dog-allergic individuals. Serum samples from 30 non-dog-allergic individuals were used as controls. The absorbance values of dog-allergic and nonallergic serum samples are reported in Figure 4D (rY-NPC2) and E (rB-NPC2). The distributions indicate that IgE from some dog-allergic individuals recognized epitopes in both rY-NPC2 and rB-NPC2. The binding of IgE from dog-allergic and non-dog-allergic individuals was found to be significantly different by the 1-tailed Mann-Whitney test ($P = .05$). In the dog-positive group, 10 (14%) of 71 serum samples had IgE binding to rB-NPC2, and 12 (17%) of 71 serum samples had IgE binding to rY-NPC2 that was greater than the 97th quantile of IgE binding in the dog-negative group. The geometric means of total IgE levels of individuals that recognized rY-NPC2 and rB-NPC2 protein is within the 95% confidence limits of geometric mean of total IgE of all 71 dog-positive individuals (data not shown).

House dust mite allergen serum pool S8Dpf was used in an IgE-inhibition experiment to evaluate whether IgE-binding epitopes are shared by dog NPC2 and dust mite allergen Der p 2. rNPC2 proteins were recognized by house dust mite serum pool S8Dpf in a dose-dependent manner (Fig 5A and B). Serum IgE antibody binding to nDer p 2 was inhibited by rY-NPC2 and rB-NPC2 proteins (11% and 9%, respectively). rY-NPC2, but not rB-NPC2, was also able to inhibit serum IgE binding to *D pteronyssinus* extract by approximately 15% (Fig 5C).

Discussion

We report identification and immunologic characterization of canine NPC2 protein, a homolog of human and bovine NPC2 proteins, as a dog allergen. Dog NPC2 has been previously reported as an epididymal protein (CE1) and a structural homolog of the human epididymis protein HE1 but not as an allergen.^{16,17} Mammalian

orthologs of NPC2 share more than 70% amino acid sequence identity and contain 6 conserved cysteine residues, 3 potential glycosylation sites, and a proline-rich region.^{15,16,18–21}

The Niemann-Pick (NP) diseases are a genotypically and phenotypically diverse group of lysosomal storage diseases that share early onset of neurologic symptoms associated with the accumulation of lipids in the central nervous system and elsewhere. NP types A and B are caused by absent or inactive sphingomyelinases, whereas the defect in NP type C is in cholesterol esterification, leading to the accumulation of free cholesterol and bis-monoacyl-glycerophosphate.¹⁵ The clinical presentation of NP type C disease is infantile hepatosplenomegaly, with neurologic symptoms appearing at 2 to 4 years, and the genotype is either a mutation in the NPC1 (18q11.2) (<http://www.omim.org/entry/607623>) or NPC2 (14q24.3) (<http://www.omim.org/entry/601015>) site.

The NPC2 family of proteins is widespread among vertebrate and invertebrate animals. Of particular interest are the group 2 allergens from 12 mite or tick species, which are NPC2 proteins specifically listed in the Allergome database (http://www.allergome.org/script/search_step2.php). As a class, these lipid-binding proteins have been implicated in the enhancement of inflammation mediated by Toll-like receptor 4 and other mechanisms.

We analyzed several dog allergen extracts by SDS-PAGE and 2D-PAGE (eTable 1). In agreement with previous investigators, we noted significant compositional differences among various dog allergenic extracts (Fig 1A)^{4,11,22,23}; the differences were more evident when proteins were separated by 2D-PAGE (data not shown). For preparation of the human serum pool, 71 dog-allergic individual serum samples were tested against dog epithelia and dog hair extracts, and we selected 51 serum samples that reacted with the tested extracts. The serum pool IgE antibodies,

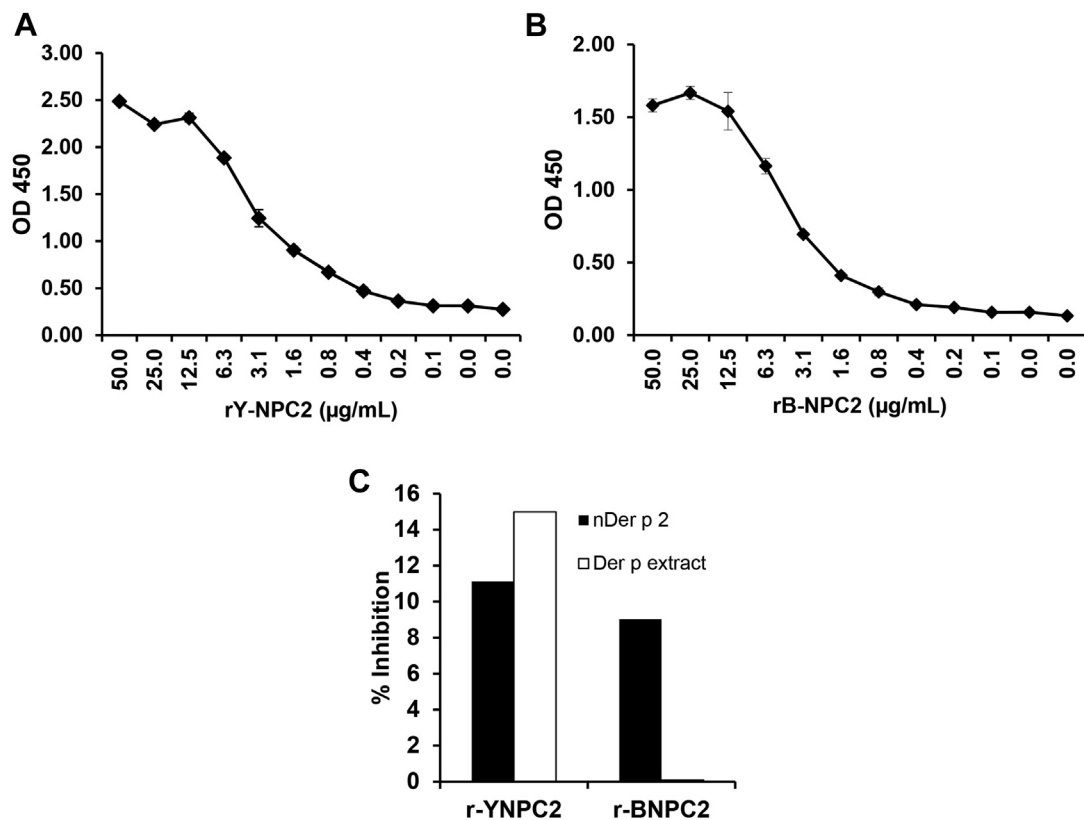


Figure 5. A and B, S8Dpf IgE binding to recombinant yeast NPC2 (rY-NPC2) and recombinant bacterial NPC2 (rB-NPC2). C, Inhibition of S8Dpf IgE binding to nDer p 2 or *Dermatophagoides pteronyssinus* allergen extract in the presence of 50 μg/mL of rY-NPC2 or rB-NPC2.

representing a proportion of the serum samples from the 51 individuals with positive test results to dog hair and epithelial extracts, recognized dog NPC2 protein in LIB-epi extract and in one of the commercial extracts (Fig 2B and D). Once the protein was identified and serum pool IgE binding was verified, the immunologic relevance of the dog NPC2 protein was evaluated using recombinant protein expressed in yeast and bacteria.

The calculated molecular weight of rY-NPC2, including hexahistidine and myc tags, is approximately 19 kDa, but the purified protein migrated with an apparent molecular mass of 26 kDa, consistent with previous observations of glycosylated NPC2.²⁴ Indeed, we observed a shift in migration on SDS-PAGE when rY-NPC2 was deglycosylated with PNGase F, indicating that rY-NPC2 is glycosylated at asparagine residues (Fig 4C). Glycosylation does not appear to affect IgE binding of NPC2; human serum pool IgE recognized rY-NPC2 and rB-NPC2 equally (Fig 4B, D, and E).

The immunologic relevance of dog NPC2 protein was estimated by measuring IgE seroprevalence against rY-NPC2 and rB-NPC2. Because our serum samples were obtained commercially and the clinical status of the individuals was not ascertained, our estimate of 10% to 20% sensitization is provisional and may differ significantly in different populations. The group 2 allergens of house dust mite belong to the ML family of proteins, sharing more than 60% sequence similarity with dog NPC2. Mechanistic and structural homology has also been reported among members of the ML family.^{25,26} The group 2 house dust mite allergens, Der p 2 and Der f 2, belong to the ML domain family of proteins, which contains 6 conserved cysteine residues that form 3 disulfide bonds.²⁶ Unlike dog NPC2 protein, mite group 2 allergens lack conserved glycosylation sites (NVS/N, NXT, and NQR/S), and the proteins only share 22% identity and 63% similarity at the amino acid level (Fig 3B). House dust mite allergic human serum pool S8Dpf recognized both rY-NPC2 and rB-NPC2 proteins in a dose-dependent manner (Fig 5A and B), and partial inhibition of binding mite-specific IgE by the recombinant NPC2 proteins was observed (Fig 5C). This finding suggests that dog NPC2 protein and group 2 house dust mite allergens contain cross-reactive determinants.

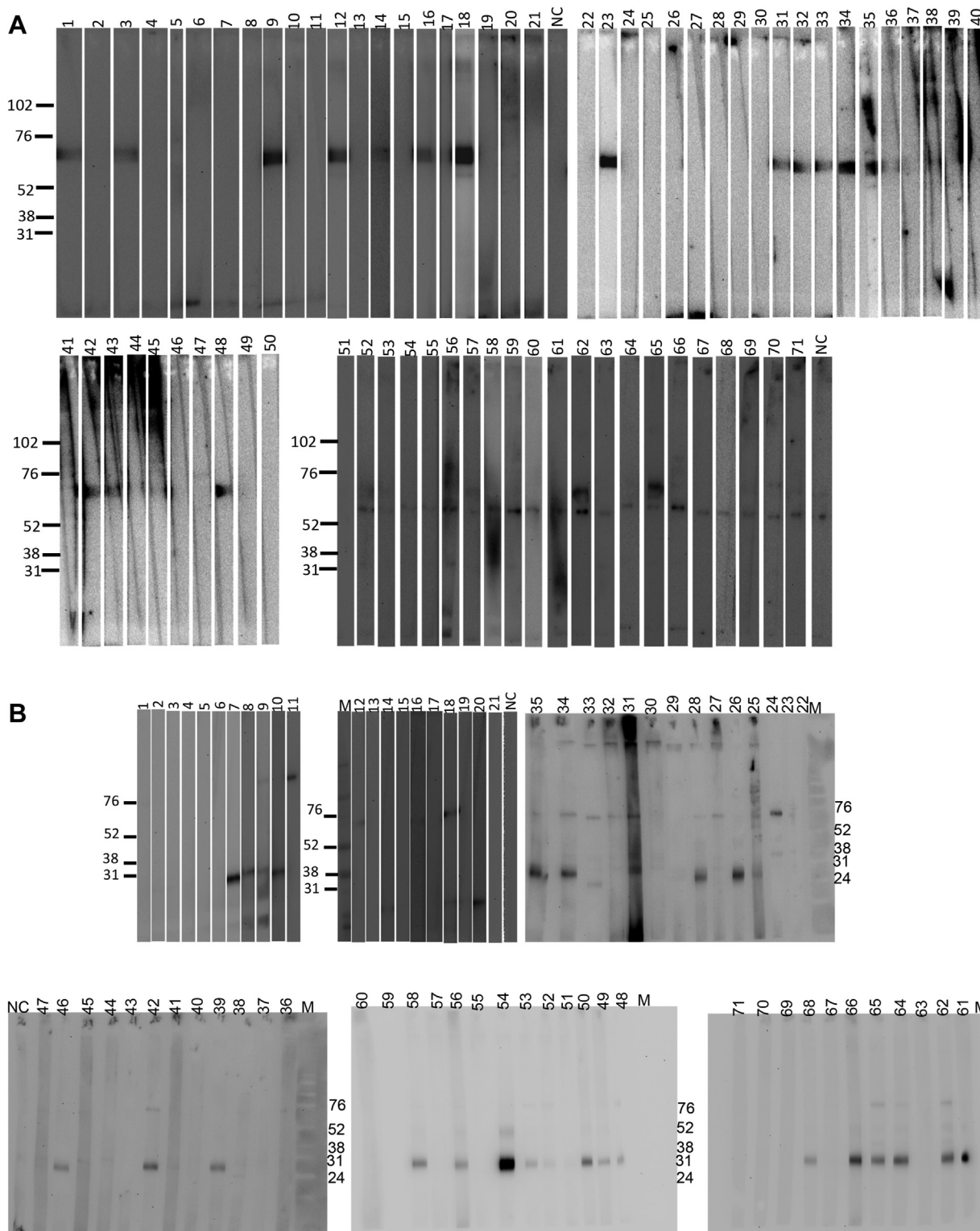
In conclusion, our findings suggest the relevance of dog NPC2 protein as new allergen for some dog-allergic individuals. Dog NPC2 protein shares sequence homology with other lipid-binding mammalian NPC2 proteins and may cross-react with group 2 dust mite allergens. The World Health Organization/International Union of Immunological Societies designation is Can f 7 (<http://allergen.org/viewallergen.php?aid=850>).

Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2016.02.006>.

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eFigure 1. Immunoblot analysis of 71 dog-allergic serum samples for reactivity against proteins in Laboratory of Immunobiochemistry dog epithelium allergen extract (A) and Laboratory of Immunobiochemistry dog hair allergen extract (B). M indicates molecular weight marker; NC, negative control.

eTable 1

Manufacturing Information About the Dog Allergen Extracts Used in This Study

Extract	Manufacturer	Source Material	Weight/Volume	Extraction Conditions	Total Protein Estimate, mg/mL
A	HollisterStier Allergy	Hair and dander	1:100	NA	2.2
B	AllerMed Laboratories	Hair	1:10	0.25% Sodium chloride, 0.125% sodium bicarbonate and 50% glycerol	3.7
C	Greer Laboratories	Hair and pelt	1:20	Coca's solution	2.2
D	ALK	Epithelia	1:10	NA	4.3
E	Allergy Labs	Hair and epithelia	1:20	NA	2.1
F	Antigen Labs	Hair	1:10	NA	0.2
LIB-hair	In house	Hair	1:10	PBS containing protease inhibitors	2.4
LIB-epi	In house	Epithelia	1:10	PBS containing protease inhibitors	1.2

Abbreviations: LIB, Laboratory of Immunobiochemistry; epi, epithelium; NA, not applicable; PBS, phosphate-buffered saline.