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Structural aspects of dog allergies: The crystal structure of a dog dander allergen Can f 4

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ABSTRACT

Four out of six officially recognized dog allergens are members of the lipocalin protein family. So far, a three-dimensional structure has been determined for only one dog allergen, Can f 2, which is a lipocalin protein. We present here the crystal structure of a second lipocalin allergen from dog, a variant of Can f 4. Moreover, we have compared and analyzed the structures of these two weakly homologous (amino acid identity 21%) dog allergens. The size and the amino acid composition of the ligand-binding pocket indicate that Can f 4 is capable of binding only relatively small hydrophobic molecules which are different from those that Can f 2 is able to bind. The crystal structure of Can f 4 contained both monomeric and dimeric forms of the allergen, suggesting that Can f 4 is able to form transient (weak) dimers. The existence of transient dimers in solution was confirmed by use of native mass spectrometry. The dimeric structure of Can f 4 is formed when the ends of four β-strands are packed against the same strands from the second monomer. The residues in the interface are mainly hydrophobic and the formation of the dimer is similar to the major horse allergen Equ c 1. Interestingly, the crystal structure of dog Can f 2 has been reported to show a different type of dimer formation. The capability of these allergens to form dimers may be important for the development of immediate allergic reaction (mast cell activation) because oligomeric allergens can effectively present multivalent epitopes.

1. Introduction

Among pets, dogs and cats are the major sources of human respiratory allergens. It has been estimated that 5–10% of the population worldwide is allergic to the domestic dog (*Canis familiaris*). Dog dander, urine and saliva contain several allergens that can induce IgE-mediated allergic reactions, such as allergic rhinitis or asthma. As allergens from dog dander and secretions can easily disperse in the environment, they are not only found in dog-owning households, but also in homes without pets as well as in public places where dogs are not usually present (Arbes et al., 2004; Morris, 2010). The development of a dog allergy is emotionally difficult for dog owners, since dogs are kept as family members. Thus, many people are not willing to give up their dogs, despite the symptoms they cause. Although some dog breeds have been suggested to be hypoallergenic and therefore suitable for allergic individuals, investigations have proven that they are as allergenic as other breeds (Nicholas et al., 2011; Vredegoor et al., 2012).

So far, six respiratory allergens have been reported for domestic dogs, namely Can f 1 to Can f 6. Can f 3 is a serum albumin (69 kDa) (Spitzauer et al., 1994). Approximately 35% of dog-allergic individuals have IgE against it and thus it is classified as a minor allergen of dog. A major allergen, mainly present in dog urine, was identified as prostatic (urine) kallikrein or arginine esterase (28 kDa). It was named Can f 5 (Mattsson et al., 2009). Up to 70% of patients with dog allergy were found to have specific IgE against Can f 5 and almost one-third was sensitized solely to Can f 5. Therefore, Can f 5 was recognized as one of the major dog allergens.

Interestingly, no less than four out of the six reported dog allergens (Can f 1, Can f 2, Can f 4, and Can f 6) are members of the lipocalin protein family. Can f 1 and Can f 2 were the first two dog

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allergens purified from dog hair and dander in 1991 (De Groot et al., 1991; Schou et al., 1991). They were later produced as recombinant proteins (Konieczny et al., 1997). Can f 1 is expressed in tongue epithelial tissue, parotid and mandibular glands, and skin. It is a major allergen, as it causes IgE antibody response in 50–70% of dog-allergic individuals. Another salivary lipocalin allergen of dog, Can f 2, sensitizes approximately one-third of dog-allergic patients and is thus a minor allergen. The IgE response of dog-sensitized individuals is often directed towards Can f 1 and Can f 2 (Konieczny et al., 1997; Saarelainen et al., 2004). A recent study indicated that a minority of patients (5%) may have Can f 2-specific IgE in the absence of Can f 1-specific IgE. Can f 2 has been suggested to be IgE cross-reactive with the cat lipocalin allergen Fel d 4. Furthermore, it was the first dog allergen for which the three-dimensional structure was solved by X-ray crystallography (Madhurantakam et al., 2010). Can f 6, which is expressed in the submaxillary gland, skin and bladder tissues of dog, was shown to sensitize 61% of dog-allergic individuals. Although Can f 6 displays low sequence identity with other dog lipocalin allergens (19–23%), its sequence similarity with the major lipocalin allergens from cat (Fel d 4, 67%) and horse (Equ c 1, 57%) is high. ELISA inhibition analyses using the sera from patients sensitized to both cat and dog indicated that Can f 6 and Fel d 4 are IgE cross-reactive (Hilger et al., 2012).

Soon after the allergenic lipocalins Can f 1 and Can f 2 were characterized, it was noticed that they were not the only dog proteins to induce IgE response. Konieczny et al. observed that three out of the 20 plasma samples examined contained IgE that bound to a dog skin test extract but did not recognize either Can f 1 or Can f 2 (Konieczny et al., 1997). Furthermore, Saarelainen et al. observed that the highest IgE reactivity in immunoblotting with a dog epithelial extract was directed against an unidentified, 18 kDa lipocalin protein presented in a dog epithelial extract (Saarelainen et al., 2004). This allergenic component was later named Can f 4. Can f 4 is a 158 amino-acid lipocalin protein expressed by tongue epithelial tissue (Mattsson et al., 2010). The allergen is principally found in saliva and dander. Can f 4 was initially purified from dog dander by Mattsson et al. and then produced as a recombinant protein in *Escherichia coli* for immunological characterization (Mattsson et al., 2010). An ImmunoCAP analysis, carried out using the sera from 37 dog-allergic patients, showed that 35% of the individuals displayed IgE against Can f 4. Moreover, one of the dog allergic patients had a strong IgE reaction to rCan f 4 without a response to other dog allergens. The results suggested that Can f 4 is a potentially significant allergen and may also act as an independent sensitizer that leads to dog allergy. Can f 4 was detected to be IgE cross-reactive with a 23 kDa odorant-binding protein isolated from cow dander, thus offering a possible linkage between dog and bovine dander allergies (Mattsson et al., 2010).

Here, we present the three-dimensional structure of the recombinant dog lipocalin allergen Can f 4, determined at a 2.6 Å diffraction resolution. The Can f 4 used in this study represents a variant in which four amino acids are different from what was previously reported (Ile20Val, Asp30Glu, Met38Leu, and Ser51Leu; Mattsson et al. (2010)). Apart from the X-ray diffraction analysis, rCan f 4 was also characterized with high-resolution mass spectrometry to verify the correctness of the expressed protein structure and to identify possible modifications. Furthermore, native mass spectrometry was used to detect the oligomeric state of the allergen in native-like solution conditions. Before this study, only one allergen structure from dog, namely the structure of the minor dog allergen Can f 2, had been determined (Madhurantakam et al., 2010). Thus, this is the first publication to describe the structure of another dog allergen, Can f 4. As the allergen is distantly homologous to Can f 2, the setting gives a possibility for structural comparisons and insights into the origin of our allergic reactivity to dogs.

2. Materials and methods

2.1. Isolation of the Can f 4 variant cDNA and the production of recombinant Can f 4

To generate the DNA fragment that encodes the Can f 4 sequence for cloning, Can f 4-specific primers were designed based on information on the canine genomic DNA sequences and the nucleotide sequence of Can f 4.

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)18 primers and AccuScript reverse transcriptase (Stratagene, LA Jolla, CA, USA) according to the supplier's instructions. The oligo(dT18) primed cDNAs were used with Can f 4 gene-specific primers (the 5′ primer includes the leader sequence of Can f 4) to amplify the fragments of the allergen by using a Phusion DNA polymerase (Finnzymes Oy, Espoo, Finland). To subclone and sequence the amplicons, products were gel-extracted by using an Ultrafree-DA Centrifugal Filter Device (Millipore, Bedford, MA, USA). Then, a Biotools DNA polymerase (Biotools, Madrid, Spain) was used to add a 3′ A overhang to purified PCR fragments for UA cloning by a Qiagen PCR cloning plus kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The nucleotide sequence of the generated construction was confirmed by DNA sequencing by using a Thermo Sequenace CY5 Dye Terminator Kit and the A.L.F Express DNA Sequencer (GE Healthcare Bioscience AB, Uppsala, Sweden). The GenBank accession number for the sequence of the full-length cDNA of the Can f 4 variant reported in this paper is KF 192077.

The achieved construct was cloned into the pPIC9 expression vector. *Pichia pastoris* yeast was transformed with the construct pPIC9rCan f 4 and the allergen expression was performed according to the manufacturer's instructions (Invitrogen, San Diego, CA, USA). The culture supernatant was clarified by centrifugation and filtration. The HiTrap NHS column (GE Healthcare) was coupled with a Can f 4 allergen-specific monoclonal antibody (48F) (see below) and the recombinant allergen was purified from the extract with the use of affinity chromatography, according to the instructions of the manufacture (GE Healthcare). Then, the rCan f 4 preparation was subjected to gel filtration on the Superdex 75 preparative grade column XK 16/70 (GE Healthcare Bioscience AB) with the GE Healthcare Äktapurifier HPLC system with PBS as the eluant. The calibration curve for the column was obtained by using a molecular-weight standard kit, MWGF70 (Sigma-Aldrich, St. Louis, USA). The rCan f 4-containing gel filtration fractions were pooled and then the buffer was exchanged for the anion-exchange chromatography with a HiTrap desalting column (GE Healthcare). Anion-exchange chromatography was performed with a Resource Q column (GE Healthcare Bioscience AB) and the bound rCan f 4 was eluted with a linear 20 CV gradient from 0 to 0.5 M NaCl in 20 mM Tris–HCl buffer at pH 7.5. The purity and IgE reactivity of the preparation was verified by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (see below). Protein concentrations were determined by absorbance spectroscopy which used the molar absorption coefficients (http://us.expasy.org/tools/protparam.html). Sterile-filtered preparations were stored at −70 °C.

2.2. Generation of the monoclonal antibody 48F specific to the Can f 4 allergen

The concentrated skin prick test extract of dog epithelium (ALK Abelló, Hørsholm, Denmark) was subjected to gel filtration, as described above. BALB/c mice were immunized intraperitoneally