

Short communication

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Phage-display reveals interaction of lipocalin allergen Can f 1 with a peptide resembling the antigen binding region of a human $\gamma\delta$ T-cell receptor

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Abstract: Although some progress has been achieved in understanding certain aspects of the allergenic mechanism of animal lipocalins, they still remain largely enigmatic. One possibility to unravel this property is to investigate their interaction with components of the immune system. Since these components are highly complex we intended to use a high-throughput technology for this purpose. Therefore, we used phage-display of a random peptide library for panning against the dog allergen Can f 1. By this method we identified a Can f 1 binding peptide corresponding to the antigen-binding site of a putative $\gamma\delta$ T-cell receptor. Additional biochemical investigations confirmed this interaction.

Keywords: binding studies; bio-panning; high-throughput assay; immunogenicity; T-cells.

Many major allergens from domestic farm and pet animals like cows, horses, dogs, cats or rodents belong to the lipocalin superfamily (Hentges et al. 2014; Hilger et al. 2012; Virtanen et al. 2012). Immunologically, lipocalin allergens have been hypothesized to reside on the border between self and foreign (Nilsson et al. 2014; Virtanen et al. 2012) as several mammalian lipocalins exhibit sequence homologies between 40 and 60% over species. Therefore, they are only weakly recognized by the adaptive immune system due to the absence of high-avidity

reactive T-cells following negative thymic selection. In turn, weak T-cell reactivity may favour a T_H2 -dominated immune response resulting in allergy development. However, although there are some new findings concerning the molecular mechanism of lipocalin allergenicity (Hufnagel et al. 2018; Klaver et al. 2019), many aspects still remain unclear. Therefore, investigations concerning the interaction of lipocalins with components of the human immune system may be helpful to elucidate their allergenic mechanism. Our idea was to use a more global approach for such an interaction screening. For this purpose, we used a phage-display based method, which was in general described to be useful in imitating the humoral immune response (Skerra 2003). As bait, we used a recombinant dog allergen Can f 1, a salivary protein present in dog hairs and dander (Konieczny et al. 1997), which is homologous to human tear lipocalin (Glasgow et al. 2002; Redl 2000). Since it is almost impossible to have all the components of the immune system represented by a phage library, a random 12-mer peptide library was used for screening. Peptide phage libraries have successfully been used in biomedical science (Wu et al. 2016). The library used in this study (NEB Ph.D.12), consists of a linear dodecapeptide and short linker sequence fused to the N-terminus of pIII of M13 phage. It contains a complexity of 10^9 independent clones, which represents only a tiny fraction of the 4.1×10^{15} possible 12-mer peptides, however. Nevertheless, this type of peptide library has proven to be of sufficient complexity to yield several DNA sequences encoding the same peptide motif. Interestingly, when panned against Taxol, the Ph.D.-12 library successfully identified peptides corresponding to the binding site of its natural ligand Bcl-2 (Rodi et al. 1999). This demonstrated the applicability of unstructured peptides to mimic three-dimensional ligand binding sites, which we sought to exploit for the identification of novel Can f 1 interacting proteins.

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We performed three rounds of biopanning for Can f 1-binding peptides and then isolated and sequenced the DNA of 10 different phages. Selection against Can f 1 resulted in the identification of two different peptides with the vast majority (9 out of 10) carrying the sequence KLWSIPTNFFLLP, while NAKYLPTILGRL was only found once. We next subjected the major peptide KLWSIPTNFFLLP to local alignment search (BLAST) against the *Homo sapiens* protein database to identify putative interacting proteins featuring these or highly similar amino acid sequences. The top 10 hits are outlined in Table 1. KLWSIPTNFFLLP was most similar to the antigen-binding CDR3 of a $\gamma\delta$ T-cell receptor isolated from human nasal mucosa (Hirata et al. 2000). Lower similarity was found to sequences from oligodendrocyte-myelin glycoprotein, a plasma membrane protein involved in neuron myelination, and enzymes of the phosphoinositide cascade.

Furthermore, we applied peptide secondary structure prediction by PEP-FOLD 3 (Shen et al. 2014) to model the structure of this peptide (Figure 1). KLWSIPTNFFLLP is likely to adopt a loop structure around P₆ which is stabilized by internal peptide backbone hydrogen bonding. Its small hydrophilic core-T₇N₈ is flanked by bulky hydrophobic side chains while charged groups are only found at the peptide termini. Its loop configuration may reflect the natural structure of the antigen-binding site in $\gamma\delta$ T-cell receptors (TCRs). Its core section -W₃S₄I₅P₆T₇N₈F₉L₁₀L₁₁- is almost identical to the CDR3 peptide's, -WGVPSNFFLL-, safe for the functionally conservative mutations I₅→V and T₇→S and the substitution of S₄ by G. From a biochemical standpoint,

both these peptides may behave very similarly. Therefore, we further investigated their interaction with Can f 1.

Due to its hydrophobic properties, we hypothesized binding of KLWSIPTNFFLLP into Can f 1's ligand cavity. Therefore, ANS (8-anilinonaphthalene-1-sulfonic acid) binding and displacement assays were used to study this supposed mode of interaction. The fluorescent probe ANS readily bound into the hydrophobic binding cavity inside Can f 1's β -barrel. An increase of specific fluorescence signal at 460 nm by more than 20-fold over free ANS signal was observed upon interaction (Figure 2A). A K_d of 2.1 μ M for ANS binding to Can f 1 was calculated, which is well within the range of lipocalin-ligand affinities previously

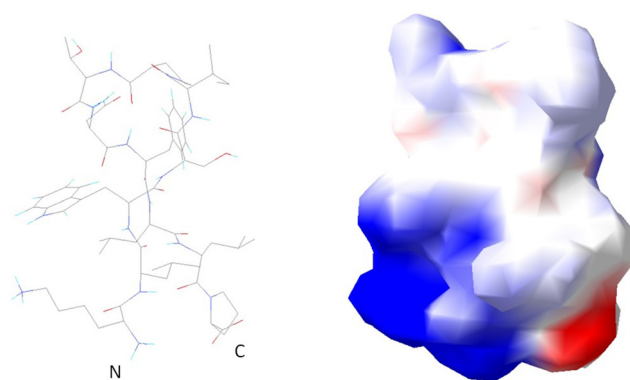


Figure 1: Secondary structure prediction (by PEP-FOLD 3): stick model and electrostatic surface potential for Can f 1-binding peptide KLWSIPTNFFLLP, derived from phage-display. Amino-(N) and carboxy-termini (C) are indicated.

Table 1: List of top 10 BLAST-hits for Can f 1 binding peptide KLWSIPTNFFLLP after searching against *Homo sapiens* database (Blastp NCBI).

#	Protein	Aligned sequence	E-value	Accession number	Localisation
1	T-cell receptor delta chain Vd1	WGVPSNFFLL	2.9	BAB16938.1	Plasma membrane
2	hCG2045700	LWSRVIPLKGNFFLLP	3	EAX01944.1	
3	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform isoform 1	SIPVDFLLP	4.1	NP_006210.1	Nucleus
4	Phosphoinositide-3-kinase, catalytic, beta polypeptide, isoform CRA_b	SIPVDFLLP	4.1	EAW79054.1	Nucleus, cytosol
5	Oligodendrocyte-myelin glycoprotein	KLWTVPTN	4.1	AAA59970.1	Plasma membrane
6	Oligodendrocyte myelin glycoprotein variant, partial	KLWTVPTN	4.1	BAD96383.1	Plasma membrane
7	Oligodendrocyte-myelin glycoprotein precursor	KLWTVPTN	4.1	NP_002535.3	Plasma membrane
8	Oligodendrocyte-myelin glycoprotein	KLWTVPTN	4.1	CAA35991.1	Plasma membrane
9	Phosphoinositide-3-kinase, catalytic, beta polypeptide, isoform CRA_c	SIPVDFLLP	4.1	EAW79056.1	Plasma membrane
10	hCG2042551, partial	IPYNFFLLP	12	EAW96483.1	

Protein name, aligned peptide sequence, expect value, NCBI accession number and subcellular localisation are indicated.

described (Breustedt et al., 2006). Therefore, the Can f 1-ANS complex was suitable for studying interaction properties between Can f 1 and the phage display-derived peptide KLWSIPTNFFLLP. For comparison, we tested the displacement of ANS from Can f 1 by increasing concentrations of palmitic acid, a typical ligand of the human Can f 1 homologue Lcn1 (Gasymov et al. 1999), which also binds to several other lipocalins (Akerstrom et al. 2000). Indeed, palmitic acid progressively replaced ANS in the binding pocket of Can f 1 (Figure 2B). The obtained ANS displacement model served as a reference to compare the influence of KLWSIPTNFFLLP-peptide on the Can f 1-ANS complex. We then tested the impact of the peptide on the fluorescence of the Can f 1-ANS complex. Surprisingly, this assay revealed a peptide concentration-dependant increase in fluorescence instead of a decrease, which is typically observed for other lipocalin ligands such as palmitic acid (Figure 2B). Intriguingly, this phenomenon was only detected when all three components—Can f 1, ANS and peptide—were present in the same sample, while peptide and ANS alone did not produce a fluorescent signal. We included two additional controls, the originally published CDR3 peptide GSWGVP SNFLLI and a scrambled control peptide (FLSPLKILT WNP, identical aa composition as the phage display peptide, but in different order), in our experiment. As depicted in Figure 2B, the CDR3 peptide produced a very similar effect as the phage display derived

peptide, while the scrambled control peptide displayed no change in signal.

These data indicate an interaction between peptide and Can f 1-ANS complex in which binding occurs without the displacement of ANS. Instead, presence of the peptide appears to change the chemical environment near the ANS molecule inside Can f 1's cavity, thereby altering the fluorescent properties of the complex. Two potential modes of interaction capable of triggering this shift include: First, a shallow binding of the peptide toward the open end of the β -barrel, which might allow for penetration of the peptide's hydrophobic side chains into the binding pocket where they contact the ANS molecule. Or second, binding to a remote site on the surface of Can f 1, which induces a conformational change affecting the ligand binding cavity.

In order to confirm the interaction of Can f 1 and the originally published δ TCR CDR3-peptide, we performed another orthogonal binding assay. For this purpose, we fused the peptide sequence SWGVPSNFFLLIVSDKLIFG (antigen-binding site in bold letters while the rear sequence is part of the TCR backbone) to the C-terminus of the SH2 domain of human intracellular phosphatase SHP-2 for expression as a fusion protein from *E. coli* (Supplementary Figure S1). This approach was chosen to avoid solubility problems due to the peptide's hydrophobic nature (Fairlie et al. 2002). The resulting fusion gene coded for

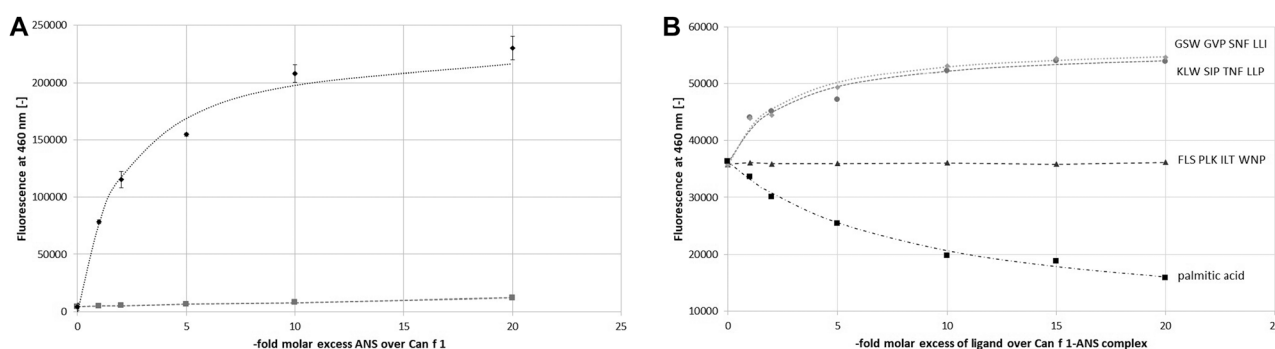


Figure 2: Fluorescence-based binding studies.

(A) Binding of ANS to Can f 1 (black). 1 μ M delipidated Can f 1 (20 μ g/ml) were mixed with a range of ANS concentrations. Samples (100 μ l per well) were prepared in triplicates and transferred to black half area 96-well microplates (Greiner Bio-One, Rainbach, Austria). Fluorescence measurements were performed on a Fluostar OMEGA plate reader (BMG Labtech, Ortenberg, Germany) with OMEGA and MARS software. Can f 1-ANS complexes were excited at 355 nm and their fluorescence recorded at 460 nm. Can f 1 binds ANS with $K_d = 2.1 \mu$ M. ANS baseline (grey). Further experimental details are given in Supplementary material.

(B) Effect of palmitic acid and different peptides on the Can f 1-ANS complex. Can f 1-ANS was titrated with increasing concentration of palmitic acid (■). Palmitic acid binds into and displaces ANS from Can f 1's ligand binding pocket. The Can f 1-ANS complex was also incubated with increasing amounts of the phage display-derived peptide KLW SIP TNF LLP (●) and the δ TCR CDR3-peptide GSW GVP SNF LLI (◆). These peptides did not displace ANS from the Can f 1-ANS complex but increased the fluorescence in a dose dependant manner. No effect of a scrambled control peptide (FLSPLKILT WNP) on the Can f 1-ANS complex was observed (▲). Measurements were done as described for panel (A). Data points represent the mean of duplicates. Experimental details are given in online Supplementary material.

a mature protein featuring an N-terminal Strep-tag, the fusion partner SH2 domain followed by the δ TCR CDR3-peptide and a C-terminal His-tag (Supplementary Figure S2).

We then used an enzyme-linked assay to study binding of the recombinant δ TCR CDR3-peptide to Can f 1. Microplates coated with Can f 1 were incubated with SWGVPSNFFLL-SH2-fusion protein. The N-terminal Strep-tag was used for detection and quantification of captured fusion protein using StrepTactin-HRP.

It is evident from Figure 3 that increased binding of SH2-CDR3 occurred in wells coated with Can f 1 in comparison to only BSA-blocked control wells. Moreover, this binding was markedly reduced by competition with a 10-fold molar excess of phage display-peptide KLWSIPTNFFLLP, indicating that binding of SH2-CDR3 to

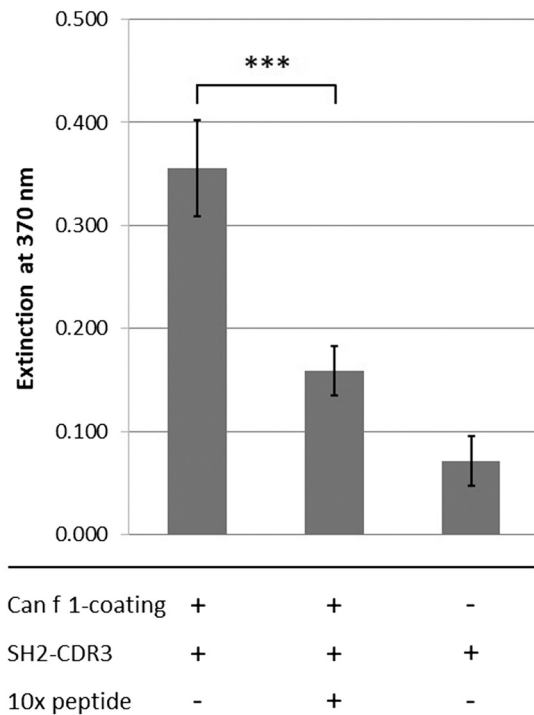


Figure 3: SH2-CDR3 fusion protein binds specifically to Can f 1-coated wells. 96-well plates were coated with Can f 1 protein and/or blocked with BSA only. SH2-CDR3 fusion protein featuring the sequence-AWGVPSNFFLL-was added in the absence or presence of a 10-fold molar excess of phage display-derived peptide KLWSIPTNFFLLP. Captured protein was measured using StrepTactin-horseradish peroxidase conjugate (SH2-CDR3 being Strep-tagged) and tetramethylbenzidine substrate. SH2-CDR3 fusion protein showed increased binding to Can f 1-coated wells compared to BSA-blocking only. This interaction was significantly inhibited ($p = 0.0038$) in the presence of KLWSIPTNFFLLP-peptide. Experiments were done in triplicates. Detailed information is given in the online Supplementary material.

Can f 1 is indeed mediated by the CDR3 peptide. Therefore, it is reasonable to assume that the δ TCR CDR3-peptide SWGVPSNFFLL may also mediate the recognition of allergenic Can f 1 protein by $\gamma\delta$ T-cells *in vivo*.

Our study supports previous indications for a role of $\gamma\delta$ T-cells in allergenicity as discussed below. In general, $\gamma\delta$ T-cells are a minor population of lymphocytes expressing the $\gamma\delta$ T-cell receptor, which constitute about 5% of T-cells in peripheral blood but as much as 50% in epithelial and mucosal tissues, where they may play an important immunological role. In contrast to $\alpha\beta$ T-cells, which canonically require antigen processing and presentation by professional antigen-presenting cells (APCs), $\gamma\delta$ T-cells are capable of directly recognizing unprocessed antigens. In fact, Rock et al. (1994) suggested ligand binding by $\gamma\delta$ T-cells to be more akin to immunoglobulins (Igs) than $\alpha\beta$ T-cell receptors (TCRs). Much like in immunoglobulins, where the heavy chain's complementary-determining region 3 (CDR3) contributes greatly to receptor diversity and represents the principal antigen binding site, the TCR δ CDR3 has been proposed as the major ligand interaction site. Since then, this assumption has been proven valid e.g. by the work of Chen et al. (2008), who successfully applied V δ 2 CDR3-derived peptides from ovarian epithelial carcinoma-infiltrating T lymphocytes for the enrichment of corresponding antigen sequences from the same Ph.D. random 12-mer peptide library applied in our study. Although only a small number of proteinaceous antigens for $\gamma\delta$ TCRs have been identified to date, it has been argued that proteins might represent the bulk of antigens recognized by $\gamma\delta$ TCRs (Born et al. 2011). Indeed, already in 1995, a study on house dust-mite (*Dermatophagoides pteronyssinus*)-allergic asthmatics (Spinozzi et al. 1995) found a pulmonary population of Der p 1 antigen-specific $\gamma\delta$ T-cells. Upon Der p 1-stimulation these cells proliferated and secreted interleukins (ILs) 4 and 5, thereby recruiting further specialized immune cells and mounting a T_H2 -type allergic response. However, the role of $\gamma\delta$ T-cells in allergic diseases is anything but clearly defined (Pawankar 2000; Zheng and Yang 2014). Although they seem to be required to elicit allergic airway inflammation in immunized, ovalbumin-challenged mice, where they produce T_H2 -type cytokines (Zuany-Amorim et al. 1998), $\gamma\delta$ T-cells have also been reported to fulfil a regulatory role by suppressing T_H2 acute airway inflammation and constraining airway remodelling (Murdoch et al. 2014). The identification of a $\gamma\delta$ TCR CDR3-derived peptide as a potential Can f 1-interacting molecule is a novel and intriguing finding and may induce further research to enlighten the the role of these cells in

lipocalin allergenicity. Thus, additional cellular studies have to be performed to proof these results *in vivo*.

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Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

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Supplementary material: The online version of this article offers supplementary material (<https://doi.org/10.1515/hsz-2020-0185>).

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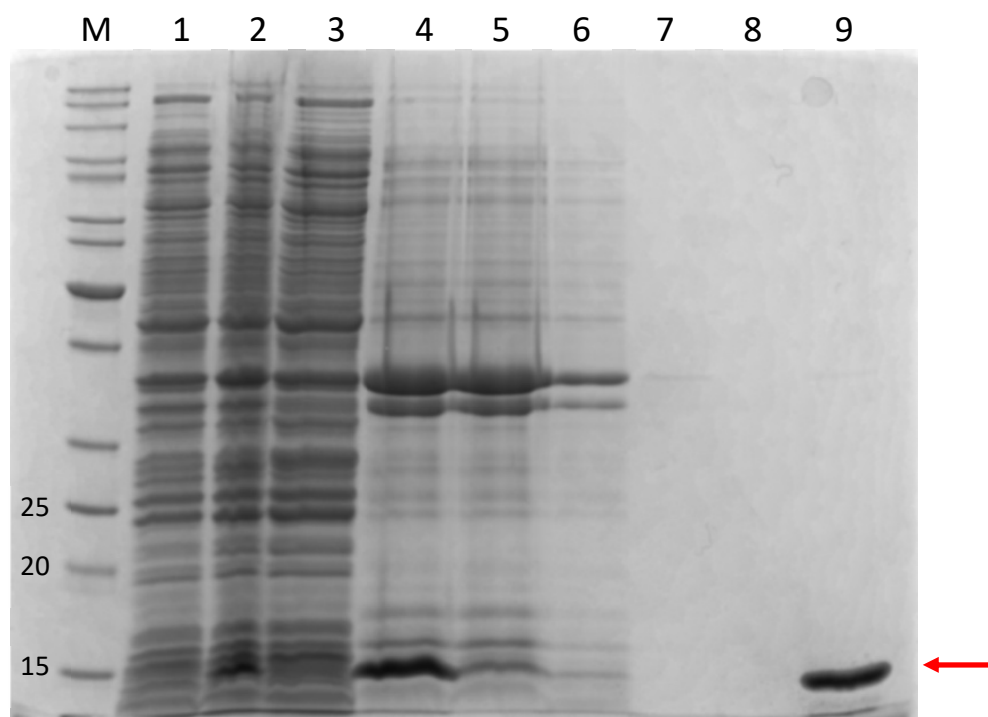
Supplementary Material

Supplementary Table S1:

#	DNA insert	Peptide
1	TCTCACTCT AAGCTTTGGTCGATTCCGACTAATTTTTGTTGCCT GGTGGAGGTTCGGCCGAA	KLWSIPTNFLLP
2	TCTCACTCT AATGCGAAGTATCTTCCGACTATCCTGGGTCGCCT GGTGGAGGTTCGGCCGAA	NAKYLPTILGRL
3	TCTCACTCT AAGCTTTGGTCGATTCCGACTAATTTTTGTTGCCT GGTGGAGGTTCGGCCGAA	KLWSIPTNFLLP
4	TCTCACTCT AAGCTTTGGTCGATTCCGACTAATTTTTGTTGCCT GGTGGAGGTTCGGCCGAA	KLWSIPTNFLLP
5	TCTCACTCT AAGCTTTGGTCGATTCCGACTAATTTTTGTTGCCT GGTGGAGGTTCGGCCGAA	KLWSIPTNFLLP
6	TCTCACTCT AAGCTTTGGTCGATTCCGACTAATTTTTGTTGCCT GGTGGAGGTTCGGCCGAA	KLWSIPTNFLLP
7	TCTCACTCT AAGCTTTGGTCGATTCCGACTAATTTTTGTTGCCT GGTGGAGGTTCGGCCGAA	KLWSIPTNFLLP
8	TCTCACTCT AAGCTTTGGTCGATTCCGACTAATTTTTGTTGCCT GGTGGAGGTTCGGCCGAA	KLWSIPTNFLLP
9	TCTCACTCT AAGCTTTGGTCGATTCCGACTAATTTTTGTTGCCT GGTGGAGGTTCGGCCGAA	KLWSIPTNFLLP
10	TCTCACTCT AAGCTTTGGTCGATTCCGACTAATTTTTGTTGCCT GGTGGAGGTTCGGCCGAA	KLWSIPTNFLLP

DNA sequences and deduced amino acid sequences of 10 Can f 1 binding phages after three rounds of panning. Peptide-coding DNA sequences are in bold letters.

Supplementary Figure S1:



Purification of Strep-SH2-CDR3-His fusion protein from *E. coli* inclusion bodies. Non-induced (1) and induced (2) sample taken during expression. Recombinant protein is absent in the soluble fraction (3) and was extracted from insoluble inclusion bodies (4). The solubilized protein was captured by Ni-NTA chromatography: Flow-through after binding (5) and washing/on column-refolding steps (washes with 4, 2 and 0.25 M urea correspond to lanes 6, 7 and 8). Purified and refolded protein (expected MW 15.9 kDa) eluted from the column (9, indicated by arrow). Molecular weight marker (M).

Supplementary Figure S2:

101	CTCAGCTTCC TTTGGGCTT TGTAGCAGC CGGATCTCAG TGGTGGTGGT GGTGGTGCTC GAGGCCGAAA ATCAGTTTGT CGCTCAGGAT CAGCAGAAAG	XhoI
	GAGTCGAAGG AAAGCCCGAA ACAATCGTCG GCCTAGAGTC ACCACCACCA CCACCACGAG CTCCGGCTTT TAGTCAAACA GCGAGTGCTA GTCGTCTTTC	
-2		*** His His His His His His Glu Leu Gly Phe Ile Leu Lys Asp Ser Val Ile Leu Leu Phe Asn
201	TTAGACGGCA CACCCACGC GAATTCGGAT CCGTTCAGAG GATATTTAAG CTCAATGACA TCTCCATTCT TCTCTTTTAA TTGCCCGTGA TGTTCATGT	EcoRI BamHI
	AATCTGCCGT GTGGGGTGGC CTTAAGCCTA GGCAAGTCCTC CTATAAATTC GAGTTACTGT AGAGGTAAGA AGAGAAAAT AACGGGCACT ACAAGGTACA	
-2		Asn Ser Pro Val Gly Trp Ala Phe Glu Ser Gly Asn Leu Pro Tyr Lys Leu Glu Ile Val Asp Gly Asn Lys Glu Lys Leu Gln Gly His His Glu Met Tyr
301	AATACTGGAC CAACTCAGCC AAAGTGGCAA ATTTCTCCCC TCCATACAGG TCATAGTAAT CACCAGTGTT CTGAATCTTG ATGTGGGTGA CAGCTCCATT	
	TTATGACCTG GTTGAGTCGG TTTCACCGTT TAAAGAGGGG AGGTATGTCC AGTATCATT GTGGTCACAA GACTTAGAAC TACACCCACT GTCGAGGTAA	
-2		Tyr Tyr Gln Val Leu Glu Ala Leu Thr Ala Phe Lys Glu Gly Gly Tyr Leu Asp Tyr Tyr Asp Gly Thr Asn Gln Ile Lys Ile His Thr Val Ala Gly Asn
401	TCTTCTAAGC GAAAGTGTGA AGTCTCCAGG GTTACTTTTA CTAGGCCTTG CCAAAAACT GCCATCACT CCTCTGTCA ACAGTAGGTT TTCTGCCTCC	
	AGAAGATTGC CTTTCACACT TCAGAGGTCC CAATGAAAAAT GATCCGGAAC GGTITTTTGA CGGTAGTTGA GGAGAACAGI TGTCATCCAA AAGACGGAGG	
-2		Arg Arg Val Ser Leu Thr Phe Asp Gly Pro Asn Ser Lys Ser Pro Arg Ala Leu Phe Ser Gly Asp Val Gly Arg Thr Leu Leu Leu Asn Glu Ala Glu Val
501	ACACCAGTGA TATTGGGTG AAACCATCTC CGCGAIGTIT ITTCAAACCTG CGGATGGCTC CAAGCCATGG TATATCTCCT TCTTAAAGTT AAACAAAATT	NcoI
	TGTGGTCACT ATAAACCCAC TTTGGTAGAG GCGCTACAAA AAAGTTTGAC GCCTACCGAG GTTCGGTACC ATATAGAGGA AGAATTTCAA TTTGTTTTAA	
-2		Val Gly Thr Ile Asn Pro His Phe Trp Arg Arg Ser Thr Lys Glu Phe Gln Pro His Ser Trp Ala Met
601	ATTTCTAGAG GGAATTGTT ATCCGCTCAC AATTCCTCTA TAGTGAGTCG TATTAATTTT CCGGGATCGA GATCTCGATC CTCTACGCCG GACGCATCGT	XbaI
	TAAAGATCTC CCCTTAACAA TAGGCGAGTG TTAAGGGGAT ATCACTCAGC ATAATTAAG CGCCCTAGCT CTAGAGCTAG GAGATGCGGC CTGCGTAGCA	

DNA sequence of the SH2-CDR3 fusion protein gene in pET21d+ backbone (encoded by reverse strand). The CDR3 peptide is given in aa position 11 to 22.

Specific materials and methods

1. Production of recombinant Can f 1.

A codon optimized Can f 1 was synthesized (GenScript, Piscataway, USA), cloned into Qiagen's pQE70 expression plasmid, produced and purified in M15 *E.coli* according to the manufacturer's instruction (QIAexpressionist).

2. Phage display:

Library: Ph.D.-12 Phage Display Peptide Library Kit E8110S (New England Biolabs GmbH, Frankfurt, Deutschland)

Materials, general handling of phages and sequencing of inserts was done as described in the Instruction Manual Ph.D Phage Display Libraries from NEB (NEB, Ipswich, USA)

Biopanning for the enrichment of lipocalin-interacting phages: Target rCan f 1 was diluted to 100 µg/mL in coating buffer (100 mM NaHCO₃ pH 8.6). 150 µL of this solution was used to coat a single well (per target protein) of a 96-wells microtiter plate (Nunc Maxisorb, Thermo Scientific, Vienna, Austria) overnight. The next morning, the coating solution was discarded and replaced by blocking buffer (5 mg/mL BSA in coating buffer). The microtiter plate was blocked for 60 min at 4 °C. Meanwhile, a 100-fold representation of the original Ph.D.-12 library was prepared by diluting 10 µL (corresponding to 10¹¹ pfu equalling 100 times the library complexity of 10⁹ individual clones) in 90 µL of TBS-T (TBS + 0.1 % Tween 20). Next, the blocking solution was discarded and the microtiter plate rapidly washed 6 times in 200 µL TBS-T buffer. The prepared phage library was added to the plate and incubated on a rotating shaker at room temperature (RT) for 60 min. Then, the phage suspension was discarded and the plate washed again 10 times in 200 µL TBS-T to remove non-binders. Finally, bound phages were eluted competitively in 100 µL solution of the respective free target (100 µg/mL Can f1 in TBS) on a rotating shaker at RT for 60 min. Panning procedure was repeated three times.

3. Binding studies

3.1. Can f 1-ANS binding studies

1 µM delipidated Can f 1 (20 µg/mL) were mixed with a range of ANS concentrations (0, 1, 2, 5, 10 and 20 µM or -fold molar excess respectively) in 20 mM potassium phosphate buffer pH 7.2 containing 0.86 % acetonitrile at RT. Acetonitrile was later used as a solvent for the synthetic peptides and was therefore already included in this experiment to rule out any adverse effects. Samples (100 µL per well) were prepared in triplicates and transferred to black half area 96-wells microplates (Greiner Bio-One, Rainbach, Austria). After 10 min incubation in the dark, fluorescence measurements were performed on a Fluostar OMEGA plate reader (BMG Labtech, Ortenberg, Germany) with OMEGA and MARS software. Can f 1- ANS complexes were excited at 355 nm and their fluorescence recorded at 460 nm. A second series of measurements omitting Can f 1 was performed under identical conditions to establish ANS background signal.

3.2. ANS displacement from Can f 1 by palmitic acid

ANS was displaced from its complex with Can f 1 (both at 1 μ M) by increasing concentrations of the hydrophobic ligand palmitic acid. A series of 50-fold palmitic acid stock solutions in EtOH was prepared – 1000, 750, 500, 250, 100 and 50 μ M as well as an EtOH blank. The reaction mixture contained 4 μ L 500 μ g/mL delipidated Can f 1, 1 μ L 100 μ M ANS, 20 mM potassium phosphate pH 7.2 (see above), 2 μ L of respective 50-fold palmitic acid stock and ddH₂O to 100 μ L. Measurements were performed as described above.

3.3. Influence of peptides on ANS binding to Can f 1

5 μ g of highly pure synthetic peptides, KLWSIPTNLLP (ChinaPeptides, Shanghai, China), scrambled control peptide FLSPLKILTWNP (ProteoGenix, Schillingheim, France) and peptide GSVGPSNLLI (ProteoGenix, Schillingheim, France) were dissolved in 30 % acetonitrile in ddH₂O at a concentration of 700 μ M. From this stock solution, a dilution series of 525, 350, 175, 70 and 35 μ M in the same solvent was made. The reaction mixture for the ANS assay contained 4 μ L 500 μ g/mL delipidated Can f 1, 1 μ L 100 μ M ANS in ddH₂O, 20 mM potassium phosphate pH 7.2, 2,85 μ L of the corresponding peptide solution, ddH₂O to 100 μ L. The final peptide concentrations were 0, 1, 2, 5, 10, 15, 20 μ M. Assays were performed as described above.

4.1. Synthesis and cloning of δ TCR CDR3 peptide and cloning into SH2-fusion protein plasmid

Synthesis of the gene encoding the δ TCR CDR3-peptide SWGVPSNLLIVSDKLIFG was performed by PCR using two codon-optimized overlapping primers, (forward: 5'-ctGGATCCatgggcagctggggtgtgccgtctaactttctgctgatcgtgagcgacaaactgatttc-3' and revers: 5'-cgCTCGAGtcattattttcaaactgcggatggtccacgcagagccgaaaatcagtttgcgctca-3'), which was then cloned into pASK75 adding a C-terminal Strep-tag onto the construct. It was afterwards subcloned into a SH2 domain containing pET21d+ plasmid. The resulting gene encoded a mature protein combined of an N-terminal Strep-tag, the SH2-fusion partner, the CDR3-peptide (slightly modified to accommodate an additional EcoRI site for control digestion) and a C-terminal His-tag (Supplementary Figure S2).

4.2. Expression, purification of SH2-CDR3 fusion peptide under denaturing conditions and renaturation

A single colony of Rosetta(DE3) cells harbouring pET21d+ SH2-CDR3 plasmid was used to inoculate 12 mL of LB medium (containing 100 μ g/mL ampicillin and 25 μ g/mL chloramphenicol). The culture was grown overnight at 37 °C on a rotating shaker (x180 rpm). The next morning, 10 mL of overnight culture were used to inoculate 250 mL of fresh LB medium (+ antibiotics). At an OD₆₀₀ of 0.5 protein expression was induced by the addition of 1 mM IPTG for 4 h. Bacteria were harvested by centrifugation at 6,000 g for 20 min and resuspended in 25 mL PBS buffer (supplemented with Roche complete protease inhibitors). Bacteria were lysed by three cycles of French Pressing (at 14,000 psi in an HTU, Digi-F-Press, Heinemann). The resulting lysate was clarified by centrifugation at 12,000 g for 20 min. Then, the supernatant was discarded and the insoluble pellet – containing the recombinant protein – was solubilised in 10 mL 8 M urea in PBS under continuous mixing at room temperature for 2 h. The extract was passed through a 1 mL bed volume Ni-NTA Agarose (Qiagen) gravity flow

column to capture the SH2-CDR3 fusion protein via its C-terminal His-tag. To allow on-column refolding, the urea concentration was reduced over the course of several washing steps: The higher urea buffer was slowly replaced by applying 5 mL of the next lower concentration carefully on top of the Ni-NTA agarose bed and allowing complete flow-through by gravity. This procedure was repeated for a total of 5 times, thereby reducing the initial 8 M urea concentration stepwise to 4, 2, 1, 0.5 and 0.25 M. The purified and refolded fusion protein was finally eluted in 3 mL PBS buffer containing 250 mM imidazole. Samples collected during protein expression, solubilisation and purification were analysed by 12 % SDS-PAGE (see Figure S1).

5. Interaction studies of Can f 1 and SH2-CDR3 fusion peptide

96-wells microtiter plates (Nunc Maxisorb, Thermo Fisher Scientific) were coated with 100 μ L of 500 μ g/mL Can f 1 in coating buffer (100 mM NaHCO₃ pH 8.6) overnight at 4 °C. The next day, the coating solution was discarded, wells briefly washed and blocked with 1 % BSA (in coating buffer). After blocking, plates were incubated with 100 μ L of 40 μ g/mL SH2- CDR3 fusion protein (diluted in PBS). In the next step, 100 μ L of StrepTactin-HRP conjugate (#2-1502-001, IBA Lifesciences, diluted 1:20.000 in PBS) were bound to captured fusion protein. Finally, plates were developed using 100 μ L of 3, 3', 5, 5'-Tetramethylbenzidine liquid substrate (TMB, Sigma) for 30 min and measured on a Fluostar OMEGA plate reader as above. A control experiment omitting Can f 1 coating was performed to determine unspecific binding and background signal. Binding of SH2-CDR3 to Can f 1-coated wells was competed against using 10-fold molar excess of the original Can f 1-binding phage-display peptide KLWSIPTNFLP. All experiments were performed in triplicates.