

Complementary DNA cloning of the predominant allergen of bovine dander: A new member in the lipocalin family

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Background: A number of allergenic proteins in animal danders have been characterized at the molecular level, but little is known of their biologic functions. We have found that the prevalence of IgE antibodies among patients with cattle-associated asthma is highest against a dander protein referred to as BDA20.

Objective: The study was performed to characterize the molecular structure of BDA20,* the predominant allergen in bovine dander.

Methods: Clones encoding allergens were identified and isolated from a complementary DNA library by immunoblotting and DNA hybridization and sequenced. Recombinant proteins were produced in *Escherichia coli*. Immunoreactivity of the recombinant proteins and amino acid sequences of peptides obtained from native BDA20 after Lys-C cleavage were used to identify clones coding for BDA20.

Results: In this article we report the cDNA and amino acid sequences of BDA20. Homology comparisons showed that BDA20 belongs to the family of lipocalins.

Conclusions: The results link a dander allergen to a group of functionally important proteins. Lipocalins are present in various body fluids and secretions of several animal species in which they function as carriers of small hydrophobic molecules, such as retinoids and pheromones. If allergenicity proves to be a property shared by lipocalins, our results will have considerable implications for allergen research. (*J Allergy Clin Immunol* 1996;97:1297-1303.)

Key words: Bovine, allergen, cDNA cloning, sequence, lipocalin, IgE binding

Animal dander and hair contain several proteins identified as allergens. Molecular properties of a number of the allergens have been described.¹ The first mammalian allergen characterized at the amino acid sequence level was the main allergen of cat (Fel d 1).² Fel d 1 is a tetrameric molecule comprising two glycosylated polypeptides of 14 to 16 kd (chain 1) and two polypeptides of 4 to 6 kd

Abbreviations used

bp:	Base pair
PAGE:	Polyacrylamide gel electrophoresis
SDS:	Sodium dodecylsulfate

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(chain 2). Chain 2 shares 28% amino acid homology with rabbit uteroglobulin. Fel d 1 is present in several tissues and secretions, but its biologic function remains unknown. In rodents, urine is another important source of allergens. The predominant allergens of mouse (Mus m 1) and rat (Rat n 1) are nonglycosylated prealbumins with a size of 17 to 20 kd.³⁻⁶ In both species the urinary concentration of the allergen is several times higher in adult males than in females. α_2 -Euglobulins of mouse and rat, the major urinary proteins, have been sequenced^{7,8} and have been shown to carry allergenic epitopes.⁹

Cow dander is the most common single cause of allergic sensitization in Finnish dairy farmers.¹⁰ Allergenic components of bovine dander have been previously characterized by biochemical and immunochemical techniques.^{11,12} We have recently isolated a panel of clones encoding allergens from a complementary DNA library of bovine skin. Our studies on the clones revealed that one of the minor allergens was identical to the oligomycin sensitivity-conferring protein of the mitochondrial adenosine triphosphate synthase complex.¹³ However, the highest prevalence of 97% of IgE antibodies among patients with cattle-associated asthma was against a protein referred to as BDA20,¹² by definition a major allergen.¹⁴

We have continued our studies on the molecular characterization of bovine allergens. In this article we report the cDNA and amino acid sequences of BDA20, the most important allergen in bovine dander. We also show that the homology comparisons warrant the admission of BDA20 to the family of lipocalins, a group of proteins functioning as carriers of small hydrophobic molecules, such as retinoids and pheromones.¹⁵

METHODS

Isolation of messenger RNA and cDNA cloning

Cow skin was homogenized mechanically in guanidinium thiocyanate buffer and used for total RNA extraction by cesium chloride centrifugation.¹⁶ Poly A RNA was purified from the total RNA with oligo dT-cellulose (Promega Corp., Madison, Wis.) and used for cDNA synthesis. Complementary DNA was cloned into the Uni ZAP XR vector (ZAP-cDNA synthesis kit; Stratagene, La Jolla, Calif.).

Immunoscreening of the cDNA library

The cDNA library was immunoscreened by using the serum of a patient with cow-induced asthma. Phage plaques, 1.4×10^5 plaque-forming units per 500 cm² plate, were lifted onto isopropyl β -D-thiogalactoside-treated nitrocellulose filters. Filters were washed, blocked by 0.1% casein in Tris-buffered saline (20 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5) for 1 hour and further incubated with the diluted (1:5) patient serum for 6 hours. Iodine 125-labeled, 1:10 diluted anti-human IgE (Phadebas RAST; Pharmacia Diagnostics, Uppsala, Sweden) was used as the secondary antibody with an overnight incubation. Filter washings and serum and conjugate dilutions were made in Tris-buffered saline with 0.05% Tween-20. After autoradiography was performed, positive plaques were selected and purified with repeated phage dilutions and immunoblottings.

Sequencing of the cDNA clones

Inserts containing Bluescript SK(−) phagemids (Stratagene) were excised from the bacteriophage Uni-ZAP XR lambda (Stratagene) and transformed to *Escherichia coli*, XL1-Blue cells (Stratagene). DNA for sequencing was purified by Wizard Minipreps DNA Purification System (Promega Corp.). Nucleotide sequencing was done with the automatic DNA sequencer A.L.F., by using an Auto Read kit (Pharmacia Biotech, Uppsala, Sweden).

Screening of the cDNA library with a DNA probe

The POT probe was a 540 base pair (bp) *Bam*HI-*Xho*I fragment isolated from the Pot4 plasmid (both of the restriction sites were from the polylinker of the vector pBluescript SK(−)). The probe was labeled with phosphorous 32-labeled deoxycytidine triphosphate by using the Multiprime DNA-labeling kit (Amersham Corp., Arlington Heights, Ill.).

Phage lifts were first prehybridized (3× saline-sodium citrate, 10× Denhardt's solution, 5 mmol/L ethylenediaminetetraacetic acid, 0.5% sodium dodecylsulfate [SDS], 0.1 mg/ml salmon sperm DNA) for 1 hour at 65° C and then hybridized overnight at 65° C in a hybridization mix (prehybridization mix plus 10% dextran sulfate and 1×10^6 cpm/ml multiprime-labeled probe). After hybridization, the filters were washed twice in 3× saline-sodium citrate, 0.5% SDS for 30 minutes at 65° C and once in 0.3× saline-sodium citrate, 0.5% SDS for 15 minutes at 65° C.

POT probe-positive plaques were picked up, and inserts were excised and sequenced as described above.

Expression vector construction, fusion protein expression, and purification

The cloning vector for expression was pGEX-2T, which expresses sequences fused to the carboxyl terminus of the glutathione transferase from *Schistosoma japonicum*.¹⁷

The generation of the insert was carried out by polymerase chain reaction. The 5' primer of TGTGG-GATCCGACTACGAGAATCCCAAGATGAAGGCCG and the 3' primer of CGATGAATTCTTATGGAGGCAATTGTCTG were used in polymerase chain reaction with Pot12 clone (see Results) to produce a DNA fragment encoding the whole open reading frame of the POT sequence with 5' and 3' cloning sites for the pGEX-2T vector. The resulting polymerase chain reaction product was isolated and digested with *Bam*HI and *Eco*RI and ligated to *Bam*HI and *Eco*RI-digested expression vector pGEX-2T according to a standard procedure.¹⁶

The recombinant expression vector, pGEX-2TPOT, was transformed into *E. coli* strain TG10B. Fusion protein expression and purification were carried out with the GST Gene Fusion System according to the manufacturer's procedures (Pharmacia Biotech).

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      10      20      30      40      50      60
      |      |      |      |      |      |
GACTACGAGAATCCCAAGATGAAGGCCGTGTTCTGACTCTCCTCTTTGGACTGGTTTGC
D Y E N P K M K A V F L T L L F G L V C

      70      80      90      100     110     120
      |      |      |      |      |      |
ACTGCCCAGGAAACTCCAGCTGAGATAGACCCCTCAAAGATTCCAGGAGAGTGGCGCATC
T A Q E T P A E I D P S K I P G E W R I

      130     140     150     160     170     180
      |      |      |      |      |      |
ATTTACGCAGCTGCAGATAACAAGGATAAGATTGTGGAAGGAGGCCCACTGAGGAATTAC
I Y A A A D N K D K I V E G G P L R N Y

      190     200     210     220     230     240
      |      |      |      |      |      |
TATCGTCGGATTGAATGTATCAATGACTGTGAATCCCTCTCCATTACATTTTATCTCAAG
Y R R I E C I N D C E S L S I T F Y L K

      250     260     270     280     290     300
      |      |      |      |      |      |
GACCAGGGCACATGCCTGTTGCTCACAGAAGTGGCAAAGAGACAAGAAGGATATGTGTAC
D Q G T C L L L T E V A K R Q E G Y V Y

      310     320     330     340     350     360
      |      |      |      |      |      |
GTCCTAGAATTTTACGGTACCAATACTTTGGAAGTGATTCATGTGTCAGAAAACATGCTG
V L E F Y G T N T L E V I H V S E N M L

      370     380     390     400     410     420
      |      |      |      |      |      |
GTAAGTTATGTTGAAAATTATGATGGGGAGAGGATCACAAAAATGACTGAAGGCCCTTGCC
V T Y V E N Y D G E R I T K M T E G L A

      430     440     450     460     470     480
      |      |      |      |      |      |
AAAGGAACCAAGTTTCACTCCAGAAGAACTTGAGAAGTATCAGCAGCTGAACAGTGAGAGG
K G T S F T P E E L E K Y Q Q L N S E R

      490     500     510     520     530     540
      |      |      |      |      |      |
GGGGTTCCAAATGAAAATATAGAAAATCTCATCAAAACAGACAATTGTCTCCATAAGAA
G V P N E N I E N L I K T D N C P P .

      550     560     570
      |      |      |
CAGTGCAGAGCGGTTGGAAAAAAAAAAAA

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FIG. 1. Nucleotide and amino acid sequence of the longest open reading frame in the cDNA clone Pot12. Numbers above the sequence indicate nucleotides. The first methionine of this open reading frame is at codon 19. The stop codon is marked with a *dot*. Amino acid sequences obtained from peptide sequencing of the native BDA20 protein are underlined.

Immunoblotting

Immunoblottings of the bovine dander extract and recombinant proteins were carried out as described previously.¹² Briefly, proteins were first separated in SDS-polyacrylamide gel electrophoresis (PAGE) (12.5% gel) with the discontinuous buffer system, and

the separated components were transferred to a nitrocellulose membrane (0.45 μ m; Millipore Corp., Bedford, Mass.). After the transfer, the nitrocellulose membrane was blocked in phosphate-buffered saline, pH 7.3, containing 0.1% casein, 0.05% Tween-20, and 0.05 wt/vol NaN_3 for 1 hour at 37° C. The membrane was cut into

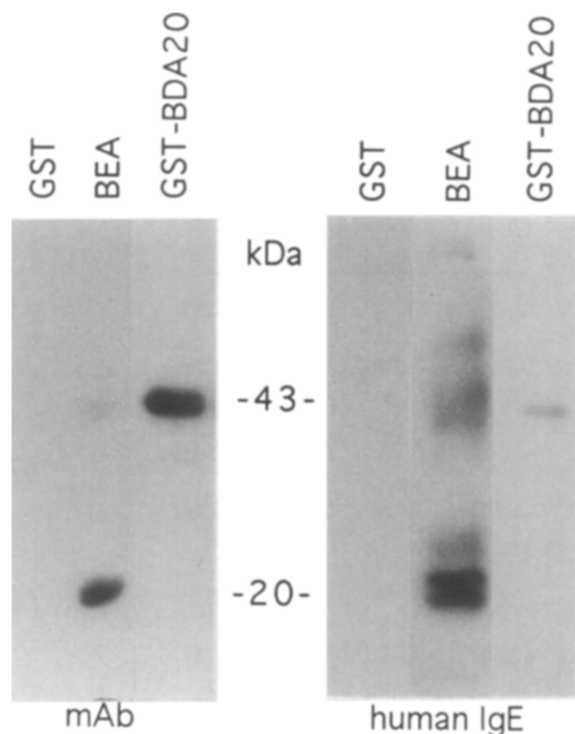


FIG. 2. Immunoblotting of the recombinant BDA20 protein expressed in *E. coli*. The slots contained glutathione transferase (GTS) expressed in *E. coli*, crude extract of bovine dander (BEA), or recombinant GST-BDA20 fusion protein (GST-BDA20). *mAb* refers to a monoclonal antibody prepared against native BDA20, and *human IgE* refers to IgE immunoblotting with the serum from a patient highly allergic to cattle.

strips and incubated overnight at room temperature with serum from a patient with cow-induced asthma (diluted 1:5) or with a monoclonal antibody (1:10 dilution of a hybridoma culture supernatant). The strips were then washed in Tween-Phosphate-buffered saline four times for 5 minutes and incubated overnight with ^{125}I -labeled anti-human IgE (Phadebas RAST) or with rabbit anti-mouse Ig, followed by ^{125}I -labeled donkey anti-rabbit Ig antiserum. After washing, the reactive bands were visualized with autoradiography.

RESULTS

DNA sequence analysis

To obtain information on the molecular structure of bovine dander allergens, we constructed a cDNA expression library from bovine skin. Several phage plaques reacting in the IgE immunoblotting were selected and purified by recloning. When the plaques of the purified clones were tested with a panel of sera from patients allergic to cattle, a high prevalence (13 of 16 sera) of IgE antibodies was found against one of the clones, Pot4. All 10 sera from nonallergic control subjects were negative. The insert of 540 bp was excised from Pot4 and

used as a probe to screen the original cDNA library. The prevalence of plaques reacting with this probe was 0.02%. An analysis of the positive plaques led to the isolation of the clone Pot12 with 51 additional base pairs at the 5' end as compared with Pot4. The sequence obtained for the longest open reading frame of Pot12 is shown in Fig. 1.

Fig. 1 also shows the amino acid sequence encoded by the longest open reading frame of 534 bp in the Pot12 clone. If the translation starts at the first methionine (codon 19), the corresponding protein would comprise 172 amino acids. This protein would have a molecular weight of 19.56 and an isoelectric point of 4.48. No putative N-linked glycosylation sites were present in the sequence.

Immunoreactivity of the recombinant protein

Our earlier results led us to the hypothesis that Pot clones correspond to the BDA20 allergen. To test this hypothesis, the open reading frame of the Pot12 clone (Fig. 1) was recloned in the pGEX-2T expression vector, and the immunoreactivity of the recombinant protein was tested in immunoblotting after PAGE. As shown in Fig. 2, both IgE antibodies in the serum of an allergic patient and a monoclonal antibody prepared against the native BDA20 allergen¹⁸ specifically recognized a protein of the expected size of 47 kD of the fusion protein. Another monoclonal antibody reactive against a different epitope in the native BDA20¹⁹ also reacted with the recombinant BDA20 (results not shown).

The reactivity of the monoclonal antibodies with the phage plaques of Pot4 (results not shown) and with the recombinant protein of the Pot12 clone suggested a relationship between this clone and the allergen BDA20 that we described earlier.¹² To confirm this, affinity-purified BDA20 was cleaved with endonuclease Lys-C, and the peptides were separated by high-pressure liquid chromatography. Peptides from two well-separated peaks were sequenced, and a partial sequence of five amino acids was obtained from a third, less well-separated peak (unpublished data). Sequences identical to the three peptides were found in the amino acid sequence corresponding to clone Pot12 (underlined in Fig. 1).

Amino acid sequence homology analysis

Sequence homology searches for the Pot12 clone were done in both the EMBL and SWISS-PROT libraries. In both cases the highest homol-

	1				50
BDA20	mkavfltlf	glvctaetp	aeidpskipG	eWRiIYaaAd	NkDKIvEGGP
pbas_rat	mrvi1111t	dvlgvssmt	dknlkkkieG	nWRTVYlaAs	svEKInEGsP
obp_rat	mvkfl1ivla	lgvscahhen	ldispsevnG	dWRTlYivAd	NvEKVaEGGs
obp_bovin		aqeee	aeqnlseIsG	pWRTVYigst	NpEKIqEnGP
aphr_crisp			..qdfaelqG	kWyTlviaAd	NlEKIeEGGP
Consensus	-----	-----	-----G	-WRT-Y--A-	N-EKI-EGGP
	51				100
BDA20	LRnYYRriEC	indCeslsit	FylKdgggtC1	llteVakrqe	gyvYvleFyG
pbas_rat	LRtYFRriEC	gkrCnrlinly	FyIKkgakCq	qfkIVgrrsq	d.vYyakYeG
obp_rat	LRaYFqhMEC	gdeCqelkii	FnVKldseCq	thtVVgqkhe	dgrYttdYsG
obp_bovin	FRtYFRelvf	ddekgtvdfy	FsVKrdgkwk	nvhVkatkqd	dgtYvadYeG
aphr_crisp	LRfYFRhiEC	yknCsemit	FyVitnnqCs	ktTVlgylkg	ngtYqtqFeG
Consensus	LR-YFR--EC	---C-----	F-VK----C-	---VV-----	---Y---Y-G
	101				150
BDA20	tNtLevihvs	enmlvtyveN	yDgeritkmt	eglakgtsft	peElekyqql
pbas_rat	staFmlktvn	ekillfdyfn	rnrrndvtrv	agvlakgrql	tkDemteymn
obp_rat	rNyFhvlkkt	ddiiffhnvN	vDesgrrqcd	lvagkredln	kaqkqelrkl
obp_bovin	qNvFkivsls	rthlvahniN	vDkhgqttel	telfvk.lnv	edEdlekfwk
aphr_crisp	nNiFqplyit	sdkiFFtnkN	mDragqetnm	ivvagkgnal	tpEeneilvq
Consensus	-N-F-----	-----N	-D-----	-----	--E-----
	151				178
BDA20	nsErgvpnen	ienlIktdnc	pP.....		
pbas_rat	fvEemgiede	nvqrVmdtdt	cPnkirir		
obp_rat	aeEynipnen	tqhlVptdtc	nq.....		
obp_bovin	ltEdkgidkk	nvvnflened	hPhpe...		
aphr_crisp	fahekkipve	nilnIlatdt	cPe.....		
Consensus	--E-----	-----	-P-----		

FIG. 3. Amino acid homology comparison of the BDA20 protein as deduced from the cDNA sequence and four lipocalins. Proteins were aligned, and consensus sequence was determined by using the CGC Sequence Analysis Software Package.²³ Amino acids 29 to 31 of BDA20 (*GEW*) represent the core of one of the highly conserved sequences of lipocalins. Lipocalins shown are rat probasin precursor (pbas_rat), rat odorant binding protein precursor (obp_rat), bovine odorant-binding protein (obp_bovin) and hamster aphrodisin (aphr_crisp).

ogies were found against representatives of a group of proteins called lipocalins.²⁰ The three proteins showing the highest degree of amino acid homology with BDA20 were rat probasin precursor (pbas_rat), rat odorant-binding protein precursor (obp_rat), and hamster aphrodisin (aphr_risp) (SWISS-PROT nos. P15399, P08937, and P09465, respectively). The amino acid identity scores between the BDA20 and the three lipocalins were 31.6%, 29.2%, and 35.3%, respectively. For comparison, the same scores were calculated between bovine odorant-binding protein, another member of the lipocalin family, and the same three lipocalins. The identity scores were 24.1%, 28.7%, and 29.3% against pbas_rat, obp_rat, and aphr_crisp, respectively. The identity scores between BDA20 and two rodent urinary proteins were 28.1% for the rat major urinary protein (mup_rat) and 25.1% for the mouse major urinary protein 1 (mup1_mouse) Fig. 3 shows a sequence comparison between BDA20 and four lipocalins. One of the two

key sequences (GXW, X usually an acidic amino acid), which is conserved in most lipocalins,^{15, 20} was found in BDA20, whereas none of the other common motifs could be identified.

DISCUSSION

In this article we present for the first time cDNA and amino acid sequences of the predominant allergen of bovine dander. We have previously estimated, from SDS-PAGE¹² and gel filtration results (unpublished observations), that the size of the native BDA20 protein is about 20 kd. The N-terminal amino acid sequence of the native BDA20 could not be determined because it was blocked. However, a comparison of the size of BDA20 and the coding capacity calculated from the first methionine to a stop codon in the cDNA clone indicates that the clone Pot12 probably represents the complete sequence of BDA20.

Our results link a mammalian dander protein with strong allergenic activity to a family of pro-

teins with important biologic functions. Lipocalins are a group of functionally related proteins from various species and sources. The level of the amino acid homology within the family is not very high, often between 10 and 20%.²⁰ However, three conserved regions have been identified in the sequence. More importantly, lipocalins all share three-dimensional structural features including a pocket that serves as a receptor for small hydrophobic molecules.²¹ Lipocalins function as carriers of these molecules, and they have been found in urine, tears, saliva, and serum. At the moment, the hypothetical molecule binding to BDA20 lipocalin remains unknown. We are using immunohistochemical techniques to determine the localization of BDA20 in bovine tissues in an attempt to discover its function.

Three bovine proteins— β -lactalbumin, odorant-binding protein, and retinol-binding protein—have been identified as lipocalins. The amino acid sequence homology of these proteins with BDA20 was not higher than that between BDA20 and lipocalins from other species. This excluded the possibility that BDA20 is identical or even closely related to these (or other previously identified) bovine proteins.

Identification of the major bovine allergen as a lipocalin raises questions about the allergenic properties of other lipocalins. One group of lipocalins that our homology search detected was the major urinary proteins of mouse and rat. These lipocalins are carriers of pheromones, and their presence in the urine is sex-dependent. α_2 -Ugoglobulins of mouse and rat, identified as the major urinary allergens,³⁻⁶ are lipocalins. If the allergenicity proves to be a general property shared by lipocalins, the situation would be similar to that of profilins. The latter are conserved proteins associated with actin polymerization in eukaryotic cells. They are also found in plants, and they are prominent allergens responsible for cross-reactive allergenicity of numerous plants.²²

Crude preparations of allergens, especially those of pollens, have been traditionally used both for diagnostic purposes and immunotherapy of allergic diseases. Amino acid sequence and molecular structure of allergens provide a rational basis for developing more specific diagnostic reagents and more effective preparations for immunotherapy. Epitope mapping is one important step in that direction. In the case of lipocalins, it is important to determine whether cross-reacting allergenic epitopes are shared by lipocalins of different origins. Ideally, then, immunotherapy with a limited

number of modified allergens or allergoids or a pool of peptides could result in immunotherapy against allergens from more than one source.

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