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Molecular cloning of canine bullous pemphigoid antigen 2 cDNA and immunomapping of NC16A domain by canine bullous pemphigoid autoantibodies[1]

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Abstract

The autoantibody-mediated subepidermal blistering skin disease bullous pemphigoid affects both humans and dogs. We previously demonstrated that canine bullous pemphigoid patient's autoantibodies targeted skin basement membrane component and a 180-kDa keratinocyte protein. We extend our works to partially isolate the cDNA encoding canine bullous pemphigoid antigen 2 (BPAg2, BP180). Total RNA extracted from a papillomavirus-immortalized canine keratinocyte cell line and a cultured canine squamous carcinoma cell line SCC 2/88 were used to isolate fragments of cDNA encoding BPAg2 by reverse transcription-PCR and 5′-rapid amplification of cDNA end. The isolated sequence included the 5′-untranslated region, the entire intracellular, transmembranous, and extracellular NC16A autoantigenic domains, plus a small segment of the collagenous domain. Sequence analyses of the isolated cDNA showed 87 and 85% identities between canine and human at the nucleotide sequence and at the deduced amino acid sequence levels, respectively. The canine BPAg2 sequence was confirmed by a rabbit antibody raised against a 18-amino acid peptide deduced from the canine NC16A nucleotide sequence. Autoantibodies from canine bullous pemphigoid patients' sera recognized epitopes within the human NC16A domain. The cloning of the cDNA encoding this disease-associated protein may allow us to develop a canine model in dissecting the immunopathologic mechanism underlying bullous pemphigoid. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Autoimmunity; Veterinary immunology; ELISA; Peptide; Cloning; Canine

1. Introduction

Bullous pemphigoid (BP) is an autoantibody-mediated subepidermal blistering skin disease characterized by IgG antibodies against the skin basement membrane zone (BMZ) BP antigens (Ags) located in the upper lamina lucida/hemidesmosome areas [1–5]. Two major Ags, the intracellular BPAg1 (BP230) and the transmembranous BPAg2 (BP180, type XVII collagen) are recognized by the patients' autoantibodies [1–3]. The human BPAg2 consists of an intracellular domain at its N-terminus, a trans-

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membranous segment, and an extracellular domain at its C-terminus [4,5]. The extracellular domain of human BPAg2 is composed of a collagenous domain, interrupted by 15 minor non-collagenous domains, at its C-terminus, and flanked by a major 77-amino acid non-collagenous domain at its N-terminus, termed NC16A [4]. This NC16A domain, located immediately C-terminal to the transmembranous segment, contains a common 14-amino acid antigenic site for the human BP autoantibodies [6]. Moreover, the antigenic epitopes of the BPAg2 are tightly clustered within a 45-amino acid stretch located at the N-terminus of this NC16A segment [7]. Whereas the pathogenic role of BPAg1 has yet to be determined, the BPAg2 has been implicated for its pathogenic role. In a cohort study of 94 BP patients, the presence of circulating autoantibodies against BPAg2, but not autoantibodies against BPAg1, correlated with a poor prognosis [8]. A more direct evidence supporting the pathogenic role of BPAg2 has been illustrated in passive transfer experiments conducted in neonatal BALB/c mice [9–11]. Rabbit antibodies raised against a recombinant protein containing the murine equivalent of human NC16A domain bound to neonatal mouse skin basement membrane and induced subepidermal blisters [9]. One of the interesting question which remains unanswered by this passive transfer model, however, is whether the eosinophil plays a role in the pathogenesis of BP blister formation [9–11].

Besides affecting human patients, BP also affects canine patients [12,13]. Like human BP, canine BP is characterized histopathologically by eosinophil infiltration in the blister cavity. We have previously demonstrated that canine BP autoantibodies labeled a BMZ component of both human and canine skin and recognized a 180-kDa protein (presumably BPAg2) present in both human and canine keratinocyte extracts [13]. The purpose of the current work was to delineate the cDNA encoding for the canine BPAg2 antigenic NC16A domain and to identify the antigenic epitopes recognized by canine BP patients' autoantibodies. Our hope is that we may be able to develop a canine animal model of BP, in order to answer the question whether eosinophil plays a role in the BP blister formation. We herein demonstrate that the 77-amino acid sequence of the BPAg2 NC16A domain in canine species shares 58% identity and 71% homology (identical plus conservatively substituted amino acids), with that of human. However, canine BP patients' autoantibodies share some immunologic similarities with that of human BP patients in that they crossreacted with antigenic epitopes on human NC16A domain that are known to be recognized by autoantibodies of human BP patients.

2. Materials and methods

2.1. Cell cultures

An immortalized canine keratinocyte cell line was established from normal dog skin. Dog skin was obtained from a healthy dog and the epidermis was removed by incubation with enzyme [14]. Dog keratinocytes were grown in the presence of a low calcium keratinocyte serum-free medium (Gibco-BRL, Grand Island, NY) [14]. When the dog keratinocytes were attached to the Petri dishes, immortalization was performed by adding papilloma virus as previously described [15]. The immortalized cells were confirmed to be keratinocytes by demonstrating the presence of keratin and BPAg2 with indirect immunofluorescence microscopy using antibodies to human keratin and BPAg2. A canine squamous cell carcinoma line was obtained from Dr. Rosol (Ohio State University, Columbus, OH). These cells were initiated from a canine oral squamous carcinoma cell line SCC 2/88 [16] and were maintained and subcultured in Williams' medium E (Gibco-BRL) containing the following supplements: 10% fetal bovine serum (Gibco-BRL), 10 ng/ml epidermal growth factor (Gibco-BRL), 0.1 nM cholera toxin (Gibco-BRL), and 2.0 mM L-glutamine (Gibco-BRL).

2.2. Isolation of cDNA fragments encoding canine BPAg2

At confluency, the papillomavirus-immortalized canine keratinocytes were washed with 1×phosphate-buffered saline (PBS) and placed on ice. The total RNA was extracted using a standardized solution RNAzol (TEL-TEST, Friendswood, TX) according to the manufacturer's manual [17]. RNA was similarly extracted from the SCC 2/88 cells which has been used by us to clone a cDNA encod-

ing a canine type VII collagen [18]. One half μg of total RNA was used in each reverse transcription (RT)-PCR reaction using an XL RNA-PCR kit specifically designed for cDNA cloning (Catalog No. N808-0205, Perkin-Elmer, Foster City, CA). Briefly, the RTs were performed in a 20-μl solution containing the RNA in the presence of 5 U of *rTth* DNA polymerase XL, 20 pmol 3′-primer, 1.1 mM $Mn(OAc)_2$, 200 μM each of dATP, dCTP, dGTP, and dTTP, and RT buffer (final concentrations: 30 mM tricine, 75 mM potassium acetate, 10% glycerol, pH 8.5). The RTs were performed at 60°C for 60 min on a GeneAmp 2400 PCR System (Perkin-Elmer). The PCR reactions were performed in a 100-μl solution containing the RT reaction mixture, plus 20 pmol 5′-primer, 1 mM $Mg(OAc)_2$, 16 μl XL RNA chelating buffer (stock concentrations: 220 mM tricine, 470 mM potassium acetate, 47% glycerol, 1.5 mM EGTA, pH 8.6). the 40-cycled PCR reaction was preceded by one cycle of denaturation (94°C, 1 min), followed by 20 cycles of 94°C, 15 s; 65°C, 3 min 45 s; and by another 20 cycles of 94°C, 15 s; and 65°C, 3 min 45 s with 15 s anneal/extension autoextension according to the manufacturer's instructions. An additional 10 min of extension (72°C) was performed after 2 U of *Taq* polymerase (Perkin Elmer) was added to the PCR solution for the purpose of creating the TA hanging. The primer pairs used in the RT-PCR experiments were designed to generate overlapping cDNA fragments using the human BPAg2 cDNA sequence as a guide (Table 1) [4,19]. When available, newly delineated canine BPAg2 cDNA sequence will be used to design primers. To isolate the nucleotide sequence of the non-coding region at the 5′-end, 5′-RACE was performed on 0.5 μg total RNA of canine SCC 2/88 cells, using a 5′-RACE kit obtained from Gibco-BRL (Version 2, Catalog No. 18374-058) and primer designed from canine BPAg2 cDNA sequence (Table 1).

2.3. cDNA cloning and sequence analysis

The RT-PCR products, determined to be single bands by electrophoresis, were ligated into TA cloning vector (Invitrogen, San Diego, CA), according to the manufacturer's manual. The ligated constructs were transformed into INVαF′ competent cells (Invitrogen) on X-Gal-coated agar plates. The white colonies were selected for plasmid DNA mini preparations and the presence of inserts was confirmed by *Eco*RI endonuclease digestion. The insert-containing clones were amplified and purified and then were subjected to double-stranded DNA sequencing by an ABI Prism Model 377 Fluorescence Automated Sequencer (Perkin-Elmer). The DNA sequence of each fragment was confirmed by four to five independently isolated TA clones. Sequence analyses, alignments, and amino acid translations were performed using MacVector and AssemblyLIGN software (Oxford Molecular, Campbell, CA).

2.4. Northern blot hybridization

Northern blot was performed by a standardized method [20], using a human cDNA probe encoding a region of the human BPAg2. Human keratinocyte

Table 1
The sequence-specific primers designed to isolate canine BPAg2 by RT-PCR and 5′-RACE

Clone no.	Primer	Nucleotide sequence (5′–3′ direction)	Size (bp)
13	RT-primer	TTTGGATCCTGCTGCAGCTGGTGGAA	767
	PCR-primer	TTCTCACCCTGGTCACCTTTGG	
8	RT-primer	GAAGAAAAACATGCCCCAGAGTC	593
	PCR-primer	ATTTTGTCCAGGTCTGCTCCCG	
2	RT-primer	ACTCAAGTGGAAGCACACGAGG	811
	PCR-primer	CTCATTCTGGACATTTGTGGTGC	
50	RT-primer	GGATCGTTGCATCGTAGGTGC	384
	PCR-primer	AGGTGGAGCCTGGGGAGTTG	

Clone 50 encodes the DNA of the 5′-non-coding end is the result of 5′-RACE, using the nesting PCR method.

Fig. 1. The canine BPAg2 molecule: nucleotide and deduced amino acid sequence. The nucleotide sequence of the 5′-end untranslated region is depicted in the first line (in lower case letters). The remaining (in capital letters) depicts the nucleotide and the deduced amino acid sequences of the coding region of the partially isolated canine BPAg2. The deduced amino acid sequence included the entire intracellular domain (amino acids 1–467), the transmembranous segment (underlined, amino acids 468–490), the extracellular NC16A domain (bracketed by **T** and **T**, amino acids 491–567), and small segment of extracellular collagenous domain (partial, amino acids 568–709) (GenBank accession no. AF016649).

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total RNA was extracted from a primary normal human keratinocyte culture as previously described [14,17]. The human BPAg2 cDNA was obtained by RT-PCR method to amplify a cDNA reversely transcribed from human keratinocyte RNA as described in the above section, using the following primer pair: RT-primer, 5′-TTCTCACCCTGGTCACCTTT-3′; PCR-primer, 5′-TCCTGCTGCAGCTGGTGGAA-3′. The human BPAg2 cDNA was cloned into TA vector. After the cDNA sequence was verified by the method described in the above section, it was then used as hybridization probe.

2.5. Anti-peptide antibody production

A peptide of 18-amino acids size was generated from amino acid sequence deduced from the isolated canine BPAg2 cDNA within the NC16A-equivalent domain: 5′-SRSNVLLFKEEMQRANKD-3′ and was coupled with KLH at the C-terminus (Sigma-Genosys, Woodlands, TX). This peptide was purified to >95% purity by HPLC and then was injected into two rabbits for anti-peptide antibodies (Sigma-Genosys). Post-immune serum containing high titer (1:25 000) of antibodies against the peptide as determined by ELISA was used in the indirect immunofluorescence and immunoblotting studies described below.

2.6. Indirect immunofluorescence studies

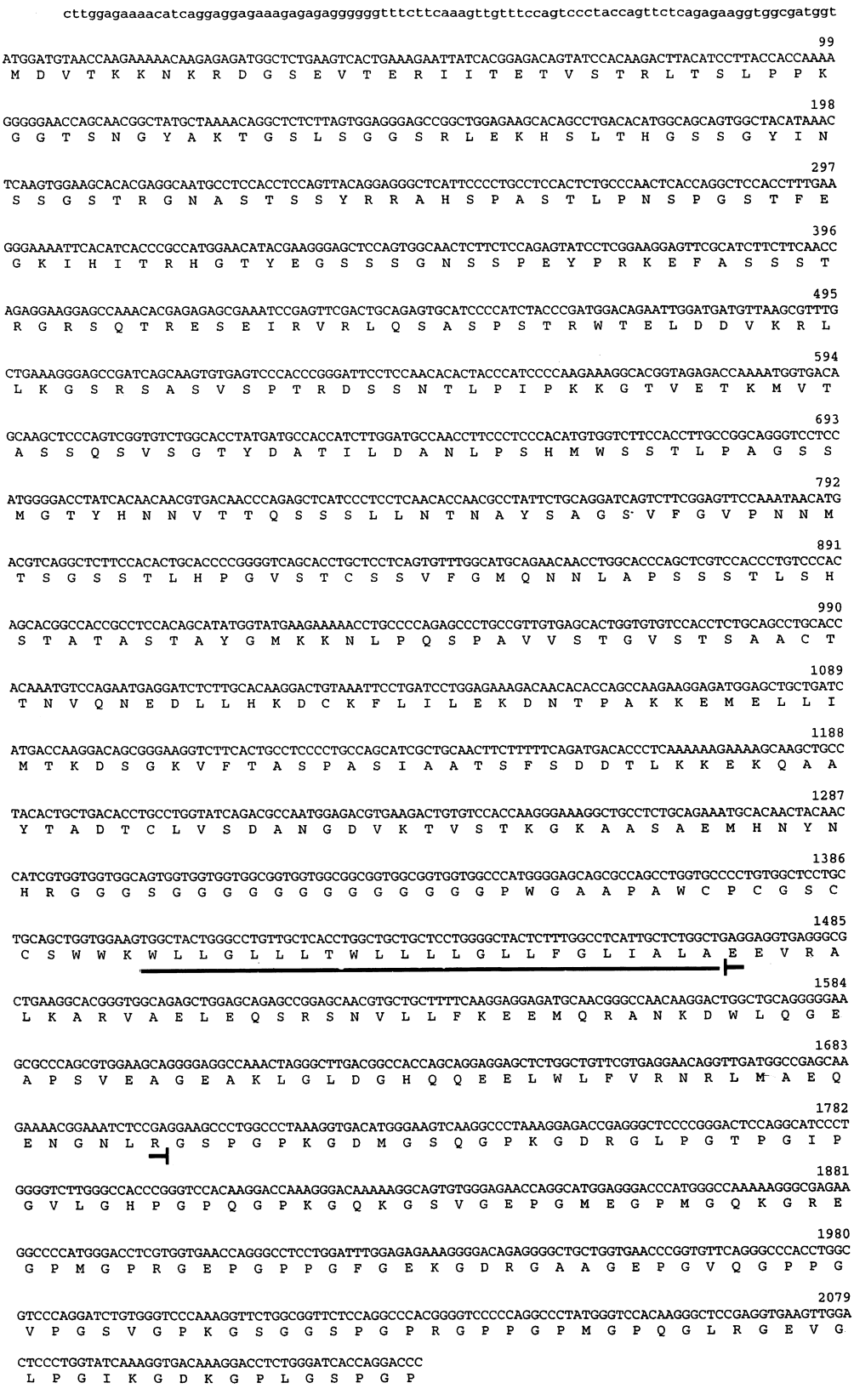
Indirect immunofluorescence was performed as previously described [21]. Six-μm-thick cryosections of salt-split normal canine skin were used as substrates [22]. In the studies with human patients' sera, the incubation of diluted sera was followed by incubation with fluorescein-conjugated goat anti-human IgG (Cappel, Durham, NC). In the studies with rabbit antisera, the incubation of diluted sera was followed by incubation of fluorescein-conjugated monoclonal mouse anti-rabbit IgG (γ-chain specific, Sigma, St. Louis, MO). Normal human serum and pre-immune rabbit serum at the same dilutions were used as negative controls. The specimens were examined under a Olympus BX60 fluorescence microscope equipped with epi-illumination and photographed.

2.7. Immunoblotting

Immunoblotting was performed as previously described [21] with slight modifications. At confluency, the SCC 2/88 cultures were first washed with 1×PBS. A mammalian culture cell protein extraction reagent (2 ml/100 mm dish, M-PER, Pierce, Rockford, IL) was added to the cell layer and the cells were placed on a shaker for 5 min at room temperature. The cell extracts were harvested by scraping followed by repeated pipetting. The supernatant was collected by centrifugation at 13 000 rpm (4°C, 10 min) and stored at −20°C. The supernatant proteins were mixed with sample buffer, boiled, loaded onto the slots of 4% stacking gel over 6% separating gel on a mini gel system of polyacrylamide gel electrophoresis (Novex, San Diego, CA), and were vertically separated at a constant voltage (125 V, 2 h),

Table 2
The non-overlapping synthetic peptides containing the entire human extracellular NC16A domain of BPAg2

Peptide no.	Amino acid sequence (5′–3′ direction)	No. of amino acids
P1	EEVRKLKARVDELE	14
P2	RIRRSILPYGDSMDRIEK	18
P3	DRLQGMAPAAGADLDKIGLHSDS	23
P4	QEELWMFVRKKLMMEQENRNLR	22


cttggagaaaacatcaggaggagaaagagagagggggttttcttcaaagttgtttccagtccctaccagttctcagagaaggtggcgatggt
99
ATGGATGTAACCAAGAAAAACAAGAGAGATGGCTCTGAAGTCACTGAAAGAATTATCACGGAGACAGTATCCACAAGACTTACATCCTTACCACCAAAA
M D V T K K N K R D G S E V T E R I I T E T V S T R L T S L P P K
198
GGGGGAACCAGCAACGGCTATGCTAAAACAGGCTCTCTTAGTGGAGGGAGCCGGCTGGAGAAGCACAGCCTGACACATGGCAGCAGTGGCTACATAAAC
G G T S N G Y A K T G S L S G G S R L E K H S L T H G S S G Y I N
297
TCAAGTGGAAGCACACGAGGCAATGCCTCCACCTCCAGTTACAGGAGGGCTCATTCCCCTGCCTCCACTCTGCCCAACTCACCAGGCTCCACCTTTGAA
S S G S T R G N A S T S S Y R R A H S P A S T L P N S P G S T F E
396
GGGAAAATTCACATCACCCGCCATGGAACATACGAAGGGAGCTCCAGTGGCAACTCTTCTCCAGAGTATCCTCGGAAGGAGTTCGCATCTTCTTCAACC
G K I H I T R H G T Y E G S S S G N S S P E Y P R K E F A S S S T
495
AGAGGAAGGAGCCAAACACGAGAGAGCGAAATCCGAGTTCGACTGCAGAGTGCATCCCCATCTACCCGATGGACAGAATTGGATGATGTTAAGCGTTTG
R G R S Q T R E S E I R V R L Q S A S P S T R W T E L D D V K R L
594
CTGAAAGGGAGCCGATCAGCAAGTGTGAGTCCCACCCGGGATTCCTCCAACACACTACCCATCCCCAAGAAAGGCACGGTAGAGACCAAAATGGTGACA
L K G S R S A S V S P T R D S S N T L P I P K K G T V E T K M V T
693
GCAAGCTCCCAGTCGGTGTCTGGCACCTATGATGCCACCATCTTGGATGCCAACCTTCCCTCCCACATGTGGTCTTCCACCTTGCCGGCAGGGTCCTCC
A S S Q S V S G T Y D A T I L D A N L P S H M W S S T L P A G S S
792
ATGGGGACCTATCACAACAACGTGACAACCCAGAGCTCATCCCTCCTCAACACCAACGCCTATTCTGCAGGATCAGTCTTCGGAGTTCCAAATAACATG
M G T Y H N N V T T Q S S S L L N T N A Y S A G S V F G V P N N M
891
ACGTCAGGCTCTTCCACACTGCACCCCGGGGTCAGCACCTGCTCCTCAGTGTTTGGCATGCAGAACAACCTGGCACCCAGCTCGTCCACCCTGTCCCAC
T S G S S T L H P G V S T C S S V F G M Q N N L A P S S S T L S H
990
AGCACGGCCACCGCCTCCACAGCATATGGTATGAAGAAAAACCTGCCCCAGAGCCCTGCCGTTGTGAGCACTGGTGTGTCCACCTCTGCAGCCTGCACC
S T A T A S T A Y G M K K N L P Q S P A V V S T G V S T S A A C T
1089
ACAAATGTCCAGAATGAGGATCTCTTGCACAAGGACTGTAAATTCCTGATCCTGGAGAAAGACAACACACCAGCCAAGAAGGAGATGGAGCTGCTGATC
T N V Q N E D L L H K D C K F L I L E K D N T P A K K E M E L L I
1188
ATGACCAAGGACAGCGGGAAGGTCTTCACTGCCTCCCCTGCCAGCATCGCTGCAACTTCTTTTTCAGATGACACCCTCAAAAAAGAAAAGCAAGCTGCC
M T K D S G K V F T A S P A S I A A T S F S D D T L K K E K Q A A
1287
TACACTGCTGACACCTGCCTGGTATCAGACGCCAATGGAGACGTGAAGACTGTGTCCACCAAGGGAAAGGCTGCCTCTGCAGAAATGCACAACTACAAC
Y T A D T C L V S D A N G D V K T V S T K G K A A S A E M H N Y N
1386
CATCGTGGTGGTGGCAGTGGTGGTGGTGGCGGTGGTGGCGGCGGTGGCGGTGGTGGCCCATGGGGAGCAGCGCCAGCCTGGTGCCCCTGTGGCTCCTGC
H R G G G S G G G G G G G G G G G G G P W G A A P A W C P C G S C
1485
TGCAGCTGGTGGAAGTGGCTACTGGGCCTGTTGCTCACCTGGCTGCTGCTCCTGGGGCTACTCTTTGGCCTCATTGCTCTGGCTGAGGAGGTGAGGGCG
C S W W K W L L G L L L T W L L L L G L L F G L I A L A E E V R A
1584
CTGAAGGCACGGGTGGCAGAGCTGGAGCAGAGCCGGAGCAACGTGCTGCTTTTCAAGGAGGAGATGCAACGGGCCAACAAGGACTGGCTGCAGGGGGAA
L K A R V A E L E Q S R S N V L L F K E E M Q R A N K D W L Q G E
1683
GCGCCCAGCGTGGAAGCAGGGGAGGCCAAACTAGGGCTTGACGGCCACCAGCAGGAGGAGCTCTGGCTGTTCGTGAGGAACAGGTTGATGGCCGAGCAA
A P S V E A G E A K L G L D G H Q Q E E L W L F V R N R L M A E Q
1782
GAAAACGGAAATCTCCGAGGAAGCCCTGGCCCTAAAGGTGACATGGGAAGTCAAGGCCCTAAAGGAGACCGAGGGCTCCCCGGGACTCCAGGCATCCCT
E N G N L R G S P G P K G D M G S Q G P K G D R G L P G T P G I P
1881
GGGGTCTTGGGCCACCCGGGTCCACAAGGACCAAAGGGACAAAAAGGCAGTGTGGGAGAACCAGGCATGGAGGGACCCATGGGCCAAAAAGGGCGAGAA
G V L G H P G P Q G P K G Q K G S V G E P G M E G P M G Q K G R E
1980
GGCCCCATGGGACCTCGTGGTGAACCAGGGCCTCCTGGATTTGGAGAGAAAGGGGACAGAGGGGCTGCTGGTGAACCCGGTGTTCAGGGCCCACCTGGC
G P M G P R G E P G P P G F G E K G D R G A A G E P G V Q G P P G
2079
GTCCCAGGATCTGTGGGTCCCAAAGGTTCTGGCGGTTCTCCAGGCCCACGGGGTCCCCCAGGCCCTATGGGTCCACAAGGGCTCCGAGGTGAAGTTGGA
V P G S V G P K G S G G S P G P R G P P G P M G P Q G L R G E V G
CTCCCTGGTATCAAAGGTGACAAAGGACCTCTGGGATCACCAGGACCC
L P G I K G D K G P L G S P G P

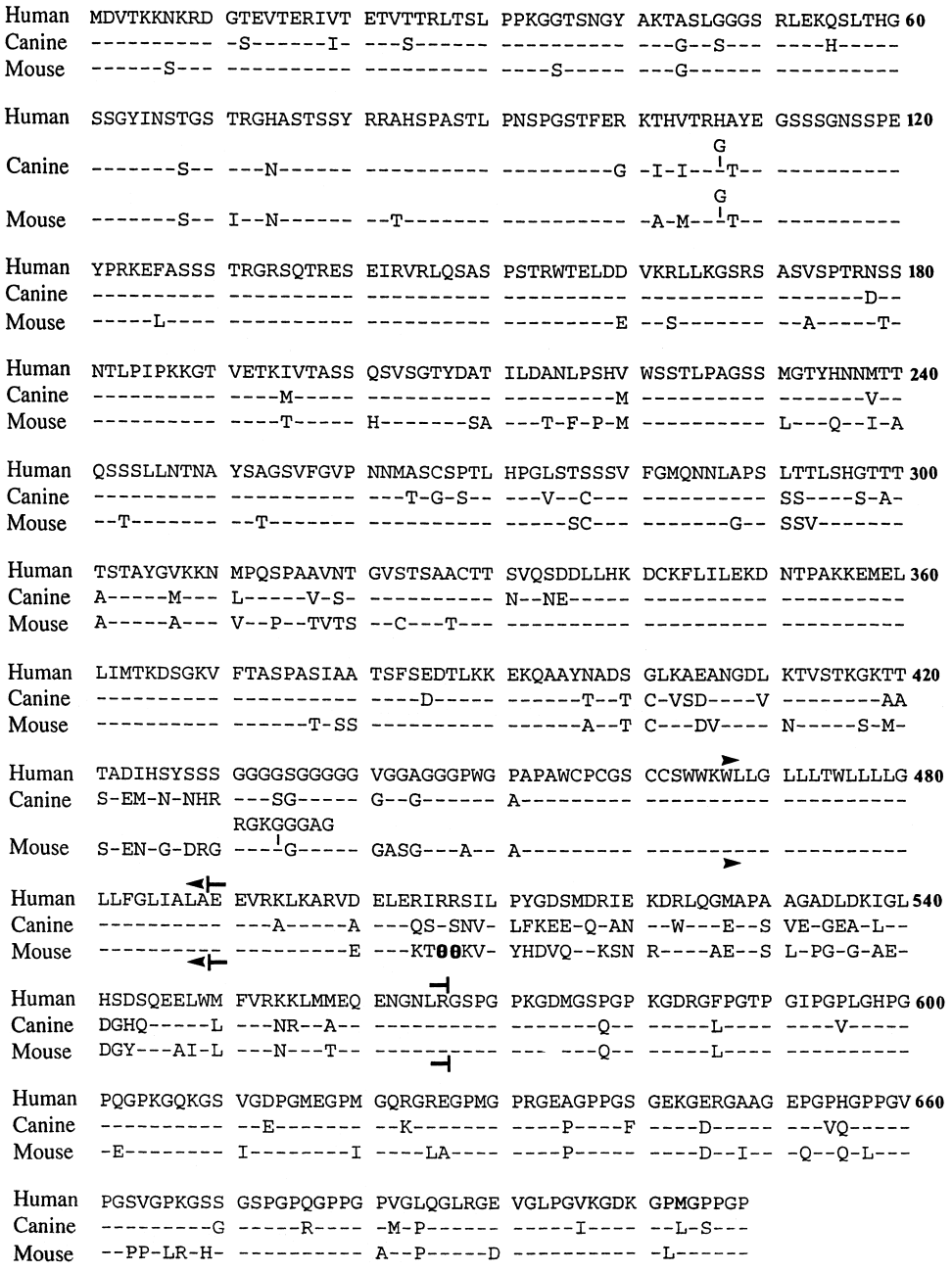


Fig. 2. Direct comparison of the human (upper line), canine (middle line), and murine (lower line) deduced amino acid sequence of the intracellular, the transmembranous, and the partial extracellular domain of BPAg2. The transmembranous domain is bracketed by ► and ►. The NC16A domain is bracketed by **T** and **T**. Two amino acids absent from the murine sequence are denoted by θ. One extra amino acid (G) is noted in the canine sequence in comparing to human sequence (amino acid 108). Extra amino acids are also noted in the murine sequence (amino acids 108 (G) and 436–443 (RGKGGGAG)). The human and murine sequences were taken directly from the GenBank database. The canine sequence has the GenBank accession no. AF016649.

under reducing conditions. The proteins were then horizontally transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). After the transfer efficiency was confirmed by Ponceau S stain (Sigma), the membranes were cut into strips, incubated in 5% powdered milk to block non-specific binding sites, and then incubated with rabbit antisera diluted in 1% BSA in Tween Tris buffer saline (Tris 50 mM,

NaCl 145 mM, 0.05% Tween-20, pH 7.5) overnight at 4°C. The immunoreactions were completed using a peroxidase-conjugated goat anti-rabbit IgG (Kirkkegaard and Perry, Gaithersburg, MD) and visualized with 4-chloro-1-naphthol (Bio-Rad).

2.8. ELISA studies

To examine whether autoantibodies from canine BP patients recognize the same antigenic epitopes that are recognized by human BP autoantibodies, ELISA studies [23] were performed with sera from two canine BP patients (one 6-year-old Weimaraner, one 2-year-old pit bull) documented by histopathology (subepidermal blister with eosinophil infiltration), immunopathology (in vivo-bound IgG deposition at skin BMZ and circulating IgG anti-BMZ autoantiboies bound to the epidermal side of salt-split skin), and immunoblotting (circulating IgG autoantibodies recognized a 180-kDa epidermal protein). Four non-overlapped peptides containing the entire major extracellular antigenic human NC16A domain were synthesized, purified by HPLC, and confirmed by mass spectrometry, and then were used in the studies (Tana Laboratories, Houston, TX, Table 2). Briefly, 5-μg peptides were coated onto each well in the 96-well plates overnight at 4°C. The diluted sera from canine BP patients and control sera (five normal dogs, a dog affected with epidermolysis bullosa acquisita), and a dog affected with linear IgA bullous dermatosis) were incubated with the peptide protein at room temperature for 3 h, followed by incubation of alkaline phosphatase-labeled goat anti-dog IgG at room temperature for 2 h. The reactions were visualized with an alkaline phosphatase substrate (Bio-Rad) and measured OD as absorbance at 405 nm wave length on an ELISA reader as previously reported [23].

2.9. Immunoelectron microscopy

Indirect immunoelectron microscopy was performed with a human BP patient's IgG anti-BPAg2 autoantibodies on salt-split canine skin using a standardized peroxidase-antiperoxidase method with some modifications [24–26]. Normal human serum at the same dilution was used as negative control. The ultrathin sections (80 nm) were examined under a Hitachi HU-12A transmission electron microscope and photographed.

3. Results and discussion

3.1. The canine BPAg2 cDNA and amino acid sequences share moderate homology to the human counterpart

Using previously delineated human BPAg2 cDNA sequence [4], primer sets were designed and utilized to isolate canine BPAg2 cDNA clones in the coding region and in the 5′-non-coding region. The double-stranded nucleotide sequence was determined by au-

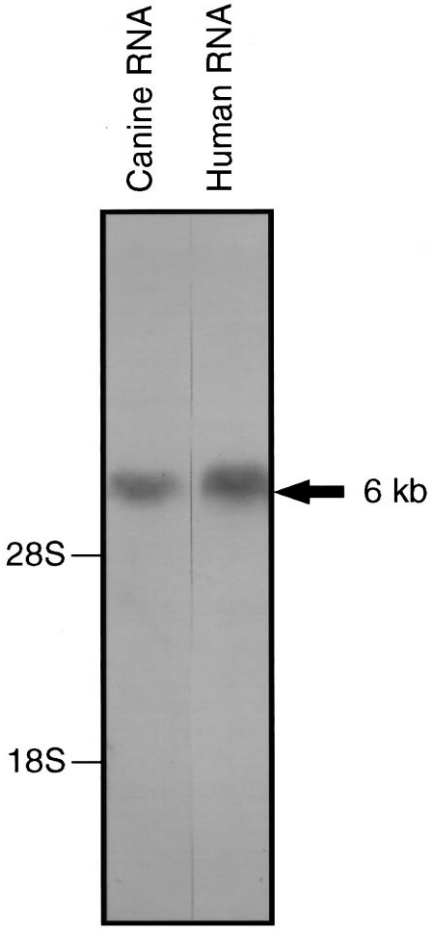


Fig. 3. The canine BPAg2 mRNA co-migrates with the human BPAg2 mRNA. Northern blot analysis of canine SSC 2/88 total RNA (left lane) with a human BPAg2 cDNA probe identifies a 6-kb mRNA (arrow), co-migrating with a 6-kb human keratinocyte mRNA (right lane) hybridized with the same cDNA probe.

Table 3
Percent amino acid identity of BPAg2 domains in human/dog and human/mouse

BPAg2 domains	Total amino acids in humans	Percent identity[a]	
		Human/dog	Human/mouse
Intracellular	466	88%	85%
Transmembrane	23	100%	100%
NC16A	77	58%	57%

[a]Identity has been calculated for amino acids of dog and mouse sequences that are identical to human sequence (absent sequence, but not extra sequences, in dog and mouse genes are included in the calculations).

tomated DNA sequencing method. The nucleotide sequence of the canine BPAg2 cDNA was submitted to GenBank database (accession number BP180 AF016649). The isolated sequence include the 5′-end of the untranslated region, the entire intracellular, transmembranous, NC16A domains, plus a small stretch of the extracellular collagenous domain. The total number of nucleotides isolated was 2127 in the coding region and 92 in the 5′-end untranslated region. Sequence analyses between the isolated canine coding sequence and the human counterpart show a 87 and 85% identity at the nucleotide and at the amino acid levels, respectively. The nucleotide sequence of the 5′-end of the untranslated region and the partially isolated coding region and its deduced amino acid sequence are shown in Fig. 1. A direct comparison of the deduced amino acid sequence (709 amino acids) of the canine BPAg2 cDNA and the human and mouse counterparts are depicted in Fig. 2. Interestingly, within the 77 amino acids of the NC16A domain, the identity between human and canine sequence is only 58% (Fig. 2), similar to the identity (57%) between the human and murine sequences in this domain [27] (Table 3). The data here showing the canine amino acid sequence at the NC16A domain being less conserved than the rest of the BPAg2 molecule is intriguing. However, this finding is not unique in canine species. Murine BPAg2 amino acid sequence, for example, shares an 81% overall identity with that of human [27]. It is also interesting in noting that the amino acid sequences of the transmembranous domains are totally identical in human, dog, and mouse genes (Fig. 2 and Table 3).

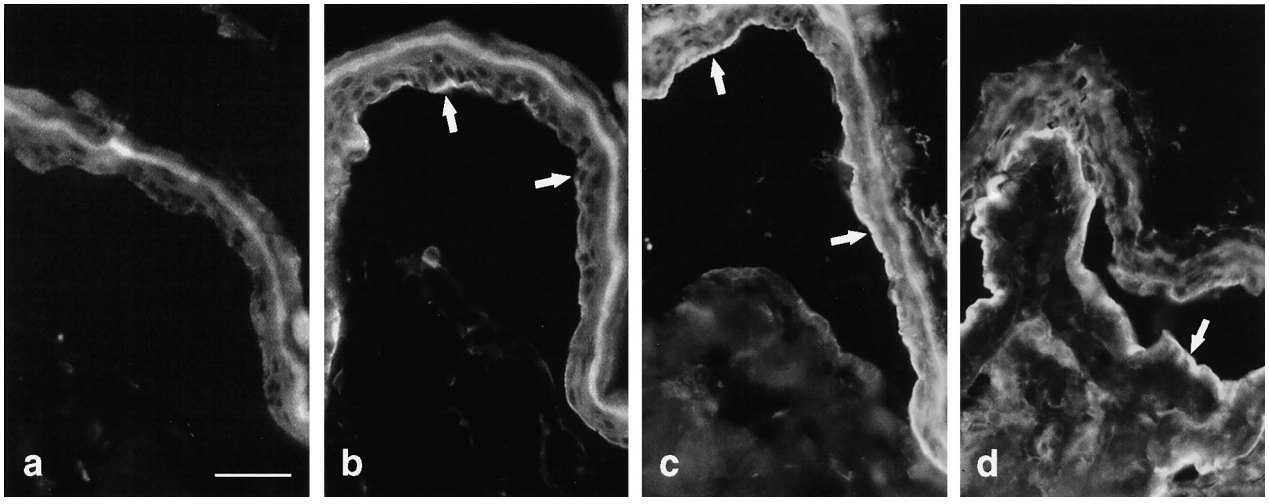


Fig. 4. The canine BPAg2 is localized to the upper skin BMZ. Indirect immunofluorescence microscopy revealed that a rabbit anti-canine NC16A peptide antiserum (b), but not the pre-immune serum from the same rabbit (a), labeled the epidermal side of BMZ on salt-split normal canine skin substrate. Control human anti-BPAg2 autoantibodies (c) and anti-type VII collagen autoantibodies (d) labeled the epidermal and dermal sides of canine salt-split skin, respectively. Arrows indicate the binding of antibodies to skin BMZ. Scale bar: 25 μm (a–d).

3.2. The canine BPAg2 mRNA co-migrates with the human BPAg2 mRNA

By Northern blot hybridization, the human BPAg2 cDNA probe hybridized a 6-kb band on SCC 2/88 total RNA. This 6-kb band co-migrated with a band from human keratinocyte total RNA hybridized with the same human BPAg2 cDNA probe (Fig. 3). These data indicate that human and canine BPAg2 mRNA share similar molecular size.

3.3. The canine BPAg2 is localized in the upper skin BMZ

By indirect immunofluorescence studies, a rabbit antibody raised against a 18-amino acid canine NC16A domain peptide labeled the epidermal side of the BMZ on salt-split normal canine skin, identical to the binding of a human BP patient's anti-BPAg2 autoantibodies; whereas a human epidermolysis bullosa acquisita patient's serum (with autoantibodies against the anchoring fibril component type VII collagen) labeled the dermal side of BMZ of canine salt-split skin (Fig. 4). These data verify that the isolated canine BPAg2 nucleotide sequence encodes a canine skin upper BMZ component. The crossreaction of human BP autoantibodies with canine skin BMZ confirms the findings previously reported [13,18] and suggests that the antigenic epitopes of human BPAg2 may be similar to that of canine BPAg2.

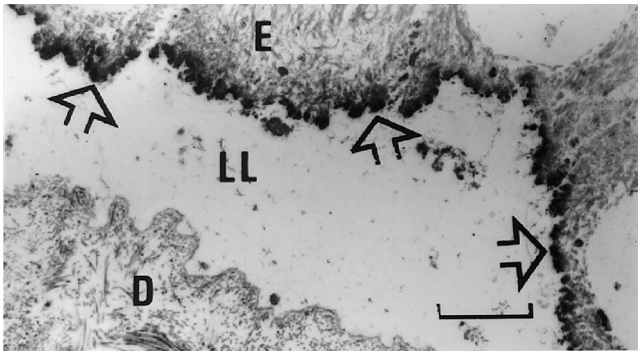


Fig. 5. The canine BPAg2 is ultrastructurally localized to the upper lamina lucida/hemidesmosome. Indirect immunoelectron microscopy performed on salt-split normal canine skin demonstrated that a human BP patient's IgG anti-BPAg2 autoantibodies labeled the BMZ upper lamina lucida/hemidesmosomes at the epidermal side (arrows). E, epidermis; D, dermis; LL, lamina lucida space separated by salt; Scale bar: 1.0 μm.

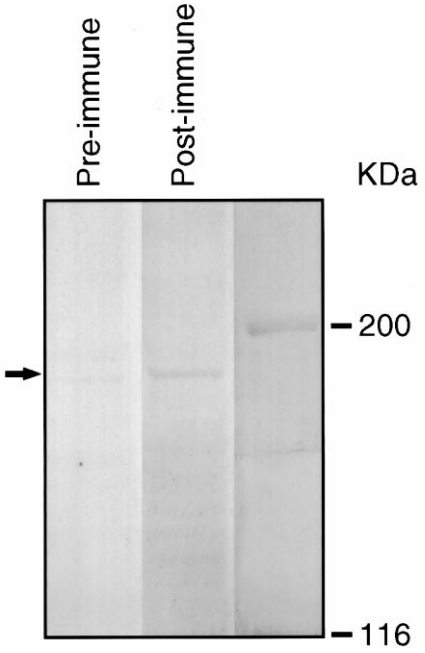


Fig. 6. The canine BPAg2 is a 180-kDa epithelial protein. Immunoblot analysis of protein extracted from canine SCC 2/88 cells revealed that a 180-kDa protein (arrow) is labeled by a rabbit antibody raised against an 18-amino acid canine NC16A peptide, but not by the same dilution of pre-immune serum from the same rabbit.

3.4. The canine BPAg2 is localized to the upper lamina lucida/hemidesmosome

Indirect immunoelectron microscopy localized human BP patient's IgG anti-BPAg2 autoantibodies binding sites exclusively to the upper lamina lucida/hemidesmosomes of canine salt-split skin (Fig. 5). No IgG was localized in the lower lamina lucida or sublamina densa. Control normal human serum did not show any IgG binding (data not shown). These data indicate that the ultrastructural location of canine BPAg2 is identical to that of human BPAg2 [24].

3.5. The canine BPAg2 is a 180-kDa epidermal protein

By immunoblotting, a rabbit antibody raised against a canine NC16A peptide, but not the pre-immune serum from the same rabbit, recognized a

180-kDa protein in the SCC 2/88 extracts (Fig. 6). Thus, these data indicate that the canine BPAg2 has a molecular size identical to that of human BPAg2 [4].

3.6. Canine BP patients' autoantibodies recognized the human antigenic NC16A epitopes

Fig. 7, upper panel, illustrates the results of ELISA studies, demonstrating that two canine BP patients' sera contained IgG autoantibodies recognizing the human NC16A domain peptides (Table 2). The IgG autoantibodies from canine patient 1 strongly recognized peptide 1, minimally reacted with peptides 2 and 4, but did not react with peptide 3. The autoantibodies from canine patient 2 strongly recognized peptide 2, moderately reacted with peptides 3 and 4, but did not react with peptide 1. The titer-dependent specificity of the autoantibodies from canine patient 1 in recognizing peptide 1 is illustrated in the lower panel of Fig. 7. Considering the rare occurrence of canine BP, we may cautiously conclude that the antigenic epitopes recognized by the canine BP autoantibodies are very similar to the epitopes recognized by human BP patients' sera as previously reported [6,7].

In this paper, we reported the isolation of a cDNA encoding for part of the canine BPAg2 that was targeted by autoantibodies from canine BP patients. Sequence analyses of the deduced amino acids of the canine NC16A domain revealed a low identity with the human NC16A domain. However, we showed that the antigenic epitopes, targeted by human BP autoantibodies, were also recognized by canine BP autoantibodies. With the antigenic epitopes of canine BP autoantibodies delineated, we can now move forward to develop a canine model of BP, in the hope that we may be able to determine the possible role of eosinophils in the BP blister formation.

While the passive transfer experiments could delineate the blistering process once the antibodies against BPAg2 are formed, another important area in need of exploration is what induces the autoantibody formation in the first place. Study of the critical initiating factors in BP is at the present time hindered by the lack of an active inducible animal model. The cloning of the cDNA encoding for this disease-targeted protein in dog may allow for the development

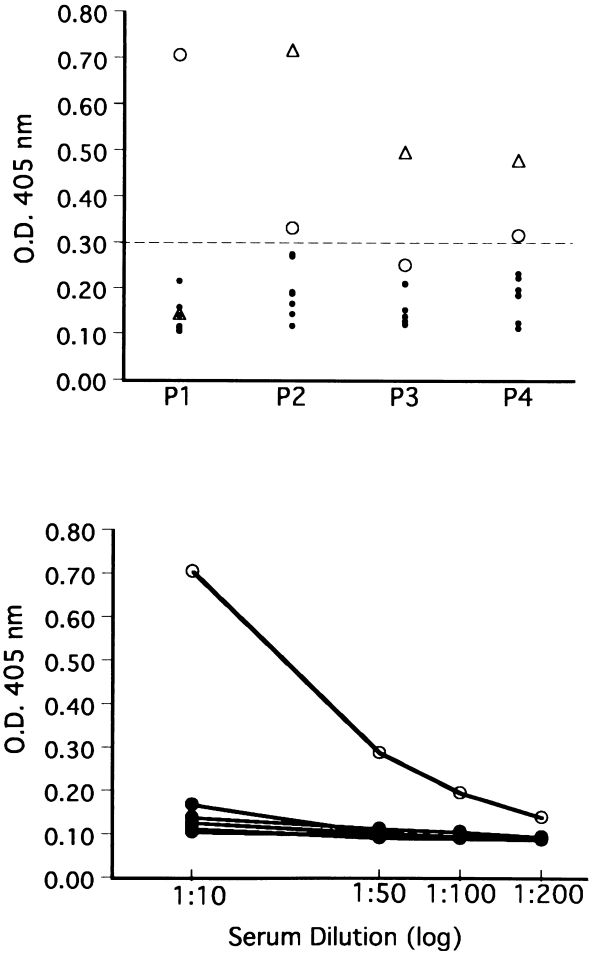


Fig. 7. The canine BP autoantibodies recognized antigenic epitopes within the human NC16A domain. Upper panel is a Scatter plot representation of ELISA results using four non-overlapping synthetic peptides containing the entire human NC16A domain (P1, P2, P3, P4). Canine patient 1 (○), canine patient 2 (△), or seven control sera (five normal dogs, one dog affected with epidermolysis bullosa acquisita, and one dog affected with linear IgA bullous dermatosis) (●). Lower panel depicts the titer-dependent specific reactivity of autoantibodies from canine patient 1 in recognizing peptide 1 of human NC16A. ELISA plate immobilized with equal amount of synthetic peptide 1 was incubated with increasing concentration of serum from canine patient 1 (○) or six control sera (●) (four normal dog, one dog each affected with epidermolysis bullosa acquisita and linear IgA bullous dermatosis). Each data point is an average of two independent observations.

of an active inducible canine BP model. While the inbred murine system provides an advantage of allowing defined immunogenetic analysis, the outbred canine system provides a different advantage in that it is clinically relevant to human disease – it is a true

reflection of the outbred nature of human patient population.

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