Molecular cloning and tissue expression of porcine β-defensin-1

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Abstract Beta-defensins constitute an emerging family of cysteine-rich antimicrobial peptides, which are particularly prominent at mucosal epithelial sites in mammals. Here we report the identification of a novel β-defensin from porcine tissues, porcine β-defensin-1 (pBD-1). The cDNA sequence of pBD-1 encoded a 64 amino acid prepro-peptide, which contained the β-defensin consensus sequence of six invariantly spaced cysteine residues. Northern blot analysis showed that pBD-1 was expressed abundantly in tongue epithelia and that the expression was regulated developmentally. Using RT-PCR, pBD-1 mRNA was detected throughout the respiratory and digestive tracts and also in thymus, spleen, lymph node, brain, liver, kidney, urinary bladder, testis, skin, heart, muscle, bone marrow, peripheral blood neutrophils, alveolar macrophages, and umbilical cord. The wide expression of pBD-1 suggests that this endogenous peptide antibiotic may contribute to both mucosal and systemic host defenses in pigs, which may have implications for the use of porcine tissues and organs in xenotransplantation.

Key words: Antimicrobial peptide; Defensin; Pig

1. Introduction

Antimicrobial peptides are important components of innate immunity [1,2]. Beta-defensins constitute a newly discovered group of cysteine-rich antimicrobial peptides with three intramolecular disulfide bonds, and many of them have been found in mammalian epithelial tissues and leukocytes of mammals and birds [3]. Based on the length and homology of peptide and gene structures, β-defensins can be grouped into three classes. The first comprises peptides with short prepro-sequences (63–64 aa) and short introns (<1.6 kbp), including bovine tracheal and lingual antimicrobial peptides (TAP, LAP [4–6]); enteric β-defensin (EBD, GenBank accession numbers AF016539 and AF000362); bovine neutrophil β-defensin 1–13 (BNBD-1–13 [7]); human β-defensin-2 (hBD-2 [8]), and sheep β-defensin-1 and 2 (sBD-1, GenBank accession number U75250, sBD-2 GenBank accession number U75251). The second comprises peptides with longer prepro-sequences (68–69 aa) and longer introns (>6.5 kbp), including hBD-1 [9]; mouse β-defensin-1 (mBD-1 [10]); and rhesus monkey β-defensin-1 (rhBD-1, GenBank accession number AF014016). The third class comprises avian β-defensins, including chicken gallinacin 1α, 1 and 2 [11,12] and turkey heterophil peptide-1 (THP-1 [12]). All of these peptides share higher identities within rather than between each group in both amino acid and nucleotide sequences. Taking advantage of the conserved cDNA sequences of these β-defensins, we identified porcine β-defensin-1 (pBD-1), a novel β-defensin that displays a wide spectrum of expression in porcine tissues and is developmentally regulated.

2. Materials and methods

2.1. Tissue and cell preparations and total RNA isolation

All tissue and cell samples were obtained from healthy outbred pigs and processed on ice within 2 h. Total RNA was extracted using TRI Reagent (Sigma, St. Louis, MO) following the manufacturer's instructions. Epithelial tissues, scraped from the tongue, urinary bladder, and different segments of the respiratory and digestive tracts, were used for RNA isolation. Tissues (~5 g) from the thymus, spleen, mesenteric lymph node, kidney, liver, lung, brain, testis, heart, muscle, and umbilical cord were collected and homogenized directly in TRI Reagent. Skin samples were dissected from underlying connective tissues before RNA isolation. Peripheral blood neutrophils and mononuclear cells were isolated from heparinized venous blood by density gradient centrifugation followed by hypotonic lysis of erythrocytes as described previously [13,14]. Bone marrow progenitor cells were obtained from the femur and fractionated by density gradient centrifugation as described above, and granulocytic lineage cells were extracted for RNA. Alveolar macrophages were obtained by bronchoalveolar lavage; washed twice with RPMI 1640 medium (Gibco BRL, Grand Island, NY); and allowed to adhere for 1 h at 39°C; only adherent macrophages were used for RNA isolation.

2.2. RT-PCR

First-strand cDNA synthesis was performed using 1.0 μg of each RNA sample primed with antisense primers (described below) in a 20 μl reaction volume containing 1 mM dNTPs; 10 units of RNasin (Perkin Elmer, Foster City, CA); and 25 units of Moloney murine leukemia virus reverse transcriptase (Perkin Elmer). All PCRs were performed with half of each resulting cDNA in a 50 μl reaction volume containing 0.2 mM dNTPs, 2 mM Mg^{2+}, 0.5 μM of each sense and antisense primer, and 1.25 units of JumpStart Taq DNA polymerase (Sigma). The primers used to obtain a 298 bp cDNA sequence of pBD-1 from tongue epithelial RNA were: sense 5′-GCCAGCATGAGGCTCCATCACCTGCTCCT-3′ (PBDP1; nucleotides 18–46 of bovine LAP cDNA sequence), and antisense 5′-AACTTTGAACAAAATTTATTCTGGTTTAAATT-3′ (PBDP2; nucleotides 286–317 of LAP cDNA). Another set of primers, which was based on the published primers for hBD-1 [15], was synthesized and also used in RT-PCR for tongue RNA. The PCR profile was: 94°C denaturation for 2 min, followed by 35 cycles of 94°C denaturation for 1 min, 60°C annealing for 1 min, and 72°C extension for 1 min, followed by a final extension at 72°C for 7 min.

Based on the pBD-1 cDNA sequence obtained from rapid amplification of cDNA ends (RACE), a new primer pair, sense 5′-ATGAGACTCCACCGCCTCCTCCT-3′ (PBDP3), and antisense 5′-GCAGCATTTGACTTGGGGCATG-3′ (PBDP4), was used in RT-PCR to determine the tissue expression of pBD-1 mRNA. Porcine genomic DNA (0.1 μg; Clontech, Palo Alto, CA) also was reverse-transcribed and amplified as described for the RNA samples. The PCR profile was the same as above except that the annealing temperature was 72°C and 40 cycles of amplification were used. All PCR products were analyzed by electrophoresis on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide.

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A.

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  1  GCCACCAGCATGAGACTCCACCGCCTCCTCCTTGTATTCCTCCTCATGGTCCTGTTACCTGTGCCAGGT   69
             M  R  L  H  R  L  L  L  V  F  L  L  M  V  L  L  P  V  P  G

 70  CTACTAAAAAACATAGGAAATTCTGTTAGCTGCTTAAGGAATAAAGGCGTGTGTATGCCGGGCAAGTGT  138
      L  L  K  N  I  G  N  S  V  S  C  L  R  N  K  G  V  C  M  P  G  K  C

139  GCTCCAAAGATGAAACAGATCGGCACCTGTGGCATGCCCCAAGTCAAATGCTGCAAAAGGAAGTAAAAA  207
      A  P  K  M  K  Q  I  G  T  C  G  M  P  Q  V  K  C  C  K  R  K  ***

208  GAAAGTGAAGAAACAACCCCACAGATATGGCTCAGAAGCTGCTCCCTTGGAAAAGCATATAAAATTTAA  276

277  ACTAGATTAAATCTTTGTTCAAGGC(A)n                                          301
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B.

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pBD-1      MRLHRLLLVFLLMVLL-PVPG--LLKNIGNSV---SCLRNKGVCMPGKCAPKMKQIGTCGMPQVKCCKRK

LAP        MRLHHLLLALLFLVLS-AGSG--FTQGVRNSQ---SCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK
TAP        MRLHHLLLALLFLVLS-AWSG--FTQGVGNPV---SCVRNKGICVPIRCPGSMKQIGTCVGRAVKCCRKK
EBD        MRLHHLLLTLLFLVLS-AGSG--FTQGISNPL---SCRLNRGICVPIRCPGNLRQIGTCFTPSVKCCRWR
BNBD-4     MRLHHLLLAVLFLVLS-AGSG--FTQRVRNPQ---SCRWNMGVCIPFLCRVGMRQIGTCFGPRVPCCRR
sBD-1      MRLHHLLLVLFFVVLS-AGSG--FTQGVRNRL---SCHRNKGVCVPSRCPRHMRQIGTCRGPPVKCCRKK
sBD-2      MRLHHLLLVLFFVVLS-AGSG--FTHGVTDSL---SCRWKKGICVLTRCPGTMRQIGTCFGPPVKCCRLK
hBD-2      MRVLYLLFSFLFIFLM-PLPG--VFGGIGDPV---TCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP

hBD-1      MRTSYLLLFTLCLLLSEMASGGNFLTGLGHRSDHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK
rhBD-1     MRTSYLLLFTLCLLLSEMASGDNFLTGLGHRSDHYNCVRSGGQCLYSACPIYTRIQGTCYHGKAKCCK
mBD-1      MKTHYFLLVMLCFLFSQMEPGVGILTSLGRRTDQYKCLQHGGFCLRSSCPSNTKLQGTCKPDKPNCCKS

Gal-1                                      GRKSDCFRKSGFCAFLKCPSLTLISGKCSRFYL-CCKRIW
THP-1                                      GKREKCLRRNGFCAFLKCPTLSVISGTCSRFQV-CC

Consensus                                    C------C----C-------G-C------CC
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Fig. 1. cDNA and peptide sequences of pBD-1 and a comparison of pBD-1 with other β-defensins. A: Nucleotide and predicted 64 amino acid sequences of pBD-1 cloned from porcine tongue. Double underline indicates the putative signal sequence, solid underline indicates the putative mature peptide, and triple asterisks indicate the stop codon. B: Comparison of prepro-pBD-1 peptide with other β-defensins. Dashes were created to optimize the alignment. Prepro-sequences of avian β-defensins (Gal-1, THP-1) are unknown. Six conserved cysteine and one glycine residues are boxed. Abbreviations: pBD-1, porcine β-defensin-1; TAP, tracheal antimicrobial peptide; LAP, lingual antimicrobial peptide; EBD, enteric β-defensin; BNBD, bovine neutrophil β-defensin; hBD, human β-defensin; sBD, sheep β-defensin; mBD-1, mouse β-defensin-1; rhBD-1, rhesus monkey β-defensin-1; Gal-1, gallinacin-1; THP-1, turkey heterophil peptide-1.

2.3. RACE

The full-length cDNA sequence of pBD-1 was obtained using a 5′- and 3′-RACE kit (Clontech). Briefly, 1 μg of porcine tongue epithelial mRNA, purified from total RNA with Oligotex (Qiagen, Chatsworth, CA), was reverse-transcribed using an oligo$(dT)_{30}$ primer, followed by second-strand cDNA synthesis using a cocktail of *Escherichia coli* DNA polymerase I, RNase H, and *E. coli* DNA ligase. The resulting double-stranded cDNA was blunted with T4 DNA polymerase and then ligated to a cDNA adapter at both ends. The 5′- and 3′-RACE products then were obtained by PCR amplification using the adapter primer and gene-specific primers from a pool of adapter-ligated double-stranded cDNAs. The gene-specific primers were PBDP4 and an oligonucleotide 5′-CTGTTACCTGTGCCAGGTCTACT-3′.

2.4. cDNA cloning and sequencing

The PCR or RACE products were cloned into pGEM-T Easy vector (Promega, Madison, WI) and then sequenced with T7 or SP6 promoter primers using a T7 Sequenase version 2.0 DNA sequencing kit (Amersham Life Science, Cleveland, OH) or sequenced directly with gene-specific primers using a Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science). All sequence reactions were performed in both directions. To obtain the partial and full-length pBD-1 cDNA sequences, more than 10 independent cDNA clones were sequenced in each case.

2.5. Southern and Northern blot analyses

For Southern analysis, PCR products from various porcine tissues were subjected to electrophoresis on 1.5% agarose gels and then capillary-transferred overnight onto nitrocellulose membranes (Micro Separations, Westboro, MA) with 20×standard saline citrate (SSC). For Northern analysis, total RNA (20 μg) was denatured and separated on 1.2% agarose/formaldehyde gels and then blotted onto nitrocellulose membranes (Micro Separations). Southern and Northern blots were prehybridized for 2 h in 5×SSC/50% formamide/0.1% SDS (Ambion, Austin, TX) at 42°C and hybridized overnight under the same conditions with ^{32}P-labeled cDNA probes produced by random priming (Pharmacia Biotech, Piscataway, NJ). Posthybridization washes were 2×10 min at 42°C with 2×SSC, followed by 2×20 min at 50°C with 0.1×SSC/0.5% SDS. Blots were exposed to Kodak X-OMAT films (Eastern Kodak, Rochester, NY) with intensifying screens at −70°C. The pBD-1 cDNA probe was generated by the PCR amplification of a 298 bp cDNA from porcine tongue epithelia using primers PBDP1 and PBDP2. A glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe was generated by PCR amplification of a 452 cDNA produced using the primers 5′-ACCACAGTCCATGCCATCAC-3′ (sense) and 5′-TCCACCACCCTGTTGCTG-3′ (antisense). Both hybridization probes (GAPDH and pBD-1) were used together. The resulting bands were evaluated by scanning densitometry, and the ratio of pBD-1 to GAPDH was calculated.

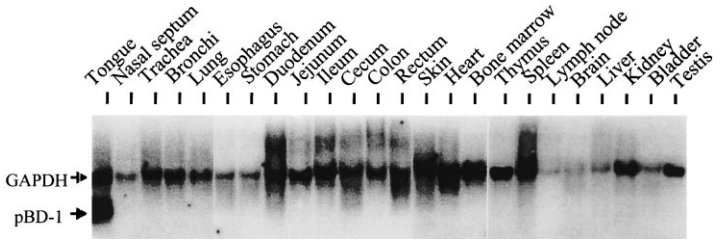

Fig. 2. Northern blot analysis of pBD-1 mRNA expression in various porcine tissue and cells from 4–5 week old pigs. 20 μg of total RNA was used per lane and probed with pBD-1 (298 bp) and GAPDH (452 bp) cDNA probes. pBD-1 mRNA was detected only in porcine tongue epithelial tissues.

3. Results

3.1. Molecular cloning of the cDNA encoding pBD-1

To identify the possible expression of β-defensins in pigs, we synthesized two different primer pairs: one was based on the highly conserved cDNA sequences of ruminant β-defensins (LAP, TAP, BNBD-4, sBD-1, and sBD-2), and the second was based on the published primers for hBD-1. A single 298 bp PCR product was found in porcine tongue using the ruminant primer pair in RT-PCR, whereas no amplified products were detected with the hBD-1 primer pair. After cloning the PCR product into the T/A vector and sequencing more than 10 independent clones, we found that they all yielded the same sequence. A new primer pair within this partial cDNA sequence was synthesized to obtain the full-length cDNA sequence using RACE techniques. Ten independent clones from each of 5′- and 3′-RACE products were sequenced, and the resulting sequence is shown in Fig. 1A. This pBD-1 cDNA is 301 bp in length and has been deposited in GenBank with accession number AF031666. The 5′ untranslated region (UTR) of this cDNA is likely not complete, because an approximate 400 bp mRNA was detected in porcine tongue by Northern blot analysis with the specific pBD-1 cDNA probe. Furthermore, the T4 DNA polymerase that was used in blunting the double-stranded cDNA may have removed 10–20 bp from the 5′ end in the RACE reaction.

The pBD-1 cDNA shared 70.0% and 70.3% identities with LAP and TAP cDNA, respectively, but only 39.9% with hBD-1 cDNA when analyzed by the Gap program of the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI). It encompassed a 192 bp open reading frame, flanked by a 9 bp 5′ UTR and a 100 bp 3′ UTR. Similar to LAP and TAP, the deduced 64 amino acid sequence of prepro-pBD-1 from the open reading frame is composed of a putative 20 residue signal sequence and a 6 residue pro-piece, followed by a mature peptide of 38 amino acid residues with a characteristic spacing pattern of six cysteines and a conserved Gly^{51} (Fig. 1B). The prepro-pBD-1 exhibited an identity of 54.7% with LAP and TAP using the Wisconsin Sequence Analysis Package (Genetics Computer Group).

3.2. Expression of pBD-1 mRNA in porcine tissues and cells

Northern blot analysis revealed expression of pBD-1 mRNA only in tongue epithelial tissues from 4–5 week old pigs (Fig. 2). However, RT-PCR analysis detected pBD-1 message throughout the epithelia of the respiratory (from nasal septum to lung) and gastrointestinal (from esophagus to rectum) tracts (Fig. 3). In addition, it was detected in nearly all organs and cells investigated, including thymus, spleen, lymph node, brain, liver, kidney, urinary bladder, testis, skin, heart, muscle, bone marrow, alveolar macrophages, peripheral blood neutrophils and the umbilical cord. The only cells evaluated that did not express pBD-1 were peripheral blood mononuclear cells. Porcine genomic DNA, processed under the same conditions as the RNA samples, gave a different size product (~1.8 kbp; data not shown).

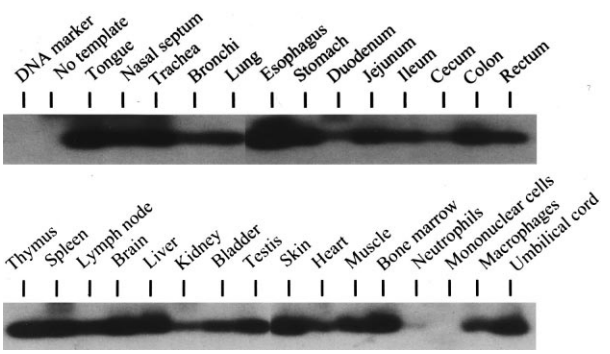

Fig. 3. Tissue expression of pBD-1 mRNA from 4–5 week old pigs by RT-PCR. 1 μg of total RNA was used, and one fourth of the 40 cycle amplified products were electrophoresed on 1.5% agarose gels, and then subjected to Southern analysis using the specific pBD-1 cDNA probe. The expected PCR product is 183 bp in length, which was found in all tissues and cells investigated except peripheral blood mononuclear cells.

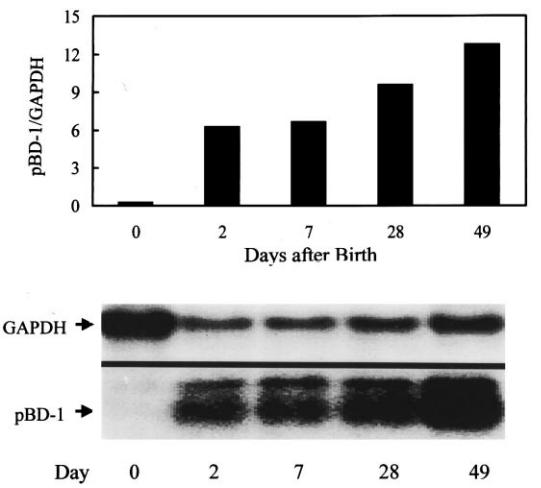

Fig. 4. Developmental expression of pBD-1 mRNA in porcine tongue epithelia. 20 μg of total RNA from littermate pigs of different ages was used per lane and probed with pBD-1 and GAPDH cDNA probes. Signals were scanned with a densitometer, and the ratio of pBD-1/GAPDH was calculated (top panel).

3.3. Developmental expression of pBD-1

To determine if pBD-1 mRNA was regulated developmentally, 20 μg of tongue epithelial RNA from littermate pigs at birth and at 2, 7, 28, and 49 days of age were subjected to Northern analysis using pBD-1 and GAPDH cDNA probes. At birth and prior to nursing (day 0), pBD-1 mRNA was virtually absent but could be detected by RT-PCR (data not shown). By 2 days of age, pBD-1 was readily detectable by Northern analysis, and its expression continued to increase postnatally (Fig. 4).

4. Discussion

Comparison of cDNA sequences of several ruminant β-defensins, including bovine LAP, TAP, EBD, BNBD-4 and sheep sBD-1 and sBD-2, revealed several conserved regions including a signal sequence encoding region and a 3′ UTR immediately preceding the poly(A) tail, which facilitated our identification of a novel β-defensin in pigs. In contrast to bovine and sheep β-defensins, no isoforms of β-defensin were found in pigs. Although no amplified PCR product was detected using primers based on the hBD-1 cDNA sequence, we cannot exclude the possibility that pigs also may express a molecule(s) similar to hBD-1, because the primer sequences that we used may not be conserved in pigs.

The deduced peptide sequence of pBD-1 reveals a very cationic peptide with two arginines, seven lysines, and no anionic residues in the mature part. A search of the non-redundant GenBank protein database (263 215 sequences as of 3 September 1997) at the National Center for Biotechnology Information using the gapped BLASTP program [16] showed that prepro-pBD-1 had significant homologies ($P < 0.01$) to 12 bovine β-defensins: LAP; TAP; EBD; and BNBD-1, 3, 4, 5, 6, 7, 9, 10, 12; two sheep β-defensins: sBD-1 and sBD-2; and one human β-defensin: hBD-2. However, the cDNA sequence of pBD-1 showed similarities only with the sheep β-defensin gene, bovine LAP and TAP, and a LAP-related cDNA when the gapped BLASTN program was used to search the GenBank gene database (303 803 sequences).

The highest level of pBD-1 mRNA was detected in tongue epithelial tissues by Northern analysis; however, expression in virtually all porcine tissue and cells was detected by the more sensitive RT-PCR. The wide expression of this molecule suggests that it may contribute to both mucosal and systemic host defenses. Similar expression patterns also have been demonstrated for human hBD-1 [15] and bovine LAP [6,17]. The expression of β-defensins in those organs that do not directly interface with a microbial environment, such as brain, liver, kidney, heart, and muscle, may facilitate defense against opportunistic infections. Alternatively, these small peptides may have other functions in those organs beyond their antimicrobial activity. Further investigations will be required to determine the cellular source and functional significance of these β-defensins in non-epithelial tissues.

In summary, a novel β-defensin, pBD-1, was identified in pigs by molecular cloning. It is expressed throughout the epithelia of the tongue and the respiratory and gastrointestinal tracts. This porcine β-defensin most closely resembles bovine and sheep β-defensins in expression pattern and cDNA and prepro-peptide sequences. Similar to other β-defensins, such as TAP [5], LAP [6], and hBD-1 [18], pBD-1 mRNA expression also was regulated developmentally and increased postnatally. To our knowledge, this is the first antimicrobial peptide of epithelial origin found in pigs. This finding will facilitate a better understanding of mucosal defense mechanisms in this species and may have important implications for the use of porcine tissues and organs in transplantation studies.

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