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Bactericidal/Permeability-Increasing Protein (BPI) and Proteinase 3: Studies at the Transcriptional Level

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A murine antibacterial ortholog to human bactericidal/permeability-increasing protein (BPI) is expressed in testis, epididymis, and bone marrow

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Abstract: The bactericidal/permeability-increasing protein (BPI), stored in human neutrophil granulocytes, is cytotoxic against Gram-negative bacteria. Several genes related to BPI cluster on human chromosome 20 and on mouse chromosome 2, but expression and characterization of a BPI ortholog in the mouse have not been reported. We asked whether BPI is structurally and functionally conserved between humans and mice and whether murine BPI might be synthesized in neutrophils as well as in other tissues. We report the isolation of a murine full-length cDNA encoding a 54-kDa protein, showing 53% amino acid identity and 71% similarity, to human BPI. The murine BPI and human BPI genes show a similar exon-intron organization. Murine BPI mRNA was detected in testis, epididymis, and bone marrow, as well as in Sertoli and promyelocytic cell lines. Although levels of BPI mRNA in human and murine testis were comparable, expression in murine bone marrow cells was low as compared with that in human bone marrow. BPI protein showed a cytoplasmic, granular localization in mature neutrophils. BPI gene expression in Sertoli and promyelocytic cells was enhanced several-fold by all-trans retinoic acid. Overexpression of murine BPI in human embryonic kidney 293 cells resulted in antibacterial activity against *Escherichia coli*, comparable with that obtained with human BPI. In conclusion, it was demonstrated that mouse neutrophils store BPI with antibacterial activity and that murine BPI is also expressed in testis and epididymis. *J. Leukoc. Biol.* 77: 369–377; 2005.

Key Words: *granulopoiesis · leukocyte · azurophil · neutrophil · antimicrobial · innate immunity*

INTRODUCTION

Antimicrobial polypeptides have an important role in the innate immune system [1]. The neutrophil granulocyte has a key role in this system as a main source of polypeptides with antibacterial activity. Other leukocytes and certain epithelial cells also produce antimicrobial polypeptides.

The bactericidal/permeability-increasing protein (BPI) is an antibacterial polypeptide synthesized during the promyelocyte stage of neutrophil differentiation for storage in azurophil (primary) granules [2] [3]. In contrast, epithelial and dermal fibroblast cells need appropriate stimulation for expression of BPI [4] [5]. At nanomolar concentrations, BPI exerts a cytotoxic and opsonic activity against Gram-negative bacteria through high-affinity binding to lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria [6]. The cationic properties of BPI, as a result of a large amount of basic amino acids in the amino-terminal half of the protein, are important for LPS binding. Binding of BPI to bacteria results in activation of endogenous bacterial phospholipase and hydrolysis of phospholipids, an increase in the permeability of the outer membrane, arrested cell division, and finally, inner membrane damage and bacteria death [7, 8]. It is interesting that although Gram-negative bacteria display variable sensitivity to BPI, the LPS-neutralizing activity of BPI is manifest with LPS also from bacteria strains resistant to antibacterial effects of BPI. Therefore, LPS neutralization and anti-inflammatory effects of BPI may be important functions of this protein [9, 10].

BPI has not been identified in mouse neutrophils [11]. However, a number of genes related to BPI cluster on chromosome 20 in humans and on chromosome 2 in the mouse. These genes encode proteins with structural similarities to BPI and include the LPS-binding protein (LBP) phospholipid transfer protein, palate, lung, and nasal epithelium (PLUNC), and RY proteins [12–16]. Although several of these proteins are predicted to have structural similarities with BPI, none of the BPI relatives in the mouse show more than 30% sequence identity to human BPI [17]. Recently, however, mouse partial transcripts with a higher sequence identity to human BPI have been available in databases, although not identified as BPI [14].

We asked whether BPI is structurally and functionally conserved between humans and mice and whether mouse BPI might be synthesized in neutrophils as well as in other tissues. To this end, we searched for mouse orthologs to human BPI. As a result, we isolated a mouse full-length cDNA encoding a 54-kDa protein, showing 53% amino acid identity and 71%

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similarity to human BPI. Mouse BPI mRNA is expressed in the testis, epididymis, and bone marrow, and BPI protein in neutrophils shows a cytoplasmic, granular localization. Moreover, overexpression of mouse BPI resulted in antibacterial activity against *Escherichia coli*, comparable with that obtained with human BPI.

MATERIALS AND METHODS

Cell culture

The TM4 Sertoli cell line [18] (European Collection of Cell Cultures, Salisbury, Wiltshire, UK, no. 88111401) was maintained in Ham's F12 Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories GmbH, Linz, Austria), supplemented with 2.5% fetal calf serum (FCS; PAA Laboratories GmbH), 5% horse serum, and 2 mM L-glutamine; the Sertoli 15P-1 cell line [19; American Type Culture Collection (ATCC), Mannassas, VA, no. CRL-2618] was maintained at 32°C in DMEM with high glucose and L-glutamine, supplemented with 10% FCS; and the mouse promyelocytic MPRO cell line [20] was maintained in DMEM, supplemented with 10% FCS and 20% HM5-conditioned medium as a source of granulocyte macrophage-colony stimulating factor. The HM5 cells, kindly provided by Dr. Jack Cowland (Granulocyte Research Laboratory, Copenhagen, Denmark), were maintained in RPMI 1640 supplemented with 10% FCS. Epithelial human embryo kidney (HEK)293 cell line, kindly provided by Thomas Hellmark (Lund University, Sweden), was maintained in DMEM/Ham'S F12 with sodium bicarbonate and L-glutamine (PAA Laboratories GmbH), supplemented with 5% FCS. In induction experiments, all-trans retinoic acid (ATRA; Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1 μM or 10 μM for the incubation times indicated. For inhibition of protein synthesis, cycloheximide was added to a final concentration of 100 μg/ml. Mouse bone marrow cells were collected from mouse C57/Bl6 femur bones, followed by lysis of erythrocytes in water for 10 s. Human bone marrow was obtained from healthy donors after informed consent; mononuclear cells were obtained by centrifugation on lymphoprep.

5′and 3′ rapid amplification of cDNA ends (RACE) and cloning of mouse BPI

Cloning of mouse BPI cDNA was performed by RACE from mouse testis, Marathon-Ready cDNA (Clontech, Palo Alto, CA), according to the manufacturer's recommendation. Primer sequences for mouse BPI were obtained from a mouse expressed sequence tag (EST; GenBank #BB584418). In 5′-RACE, the adapt primer was 5′-GCCACATTACCACCTTTTGCTCCAACTGC-3′ and nested primer was 5′-GCATCCAGGGCGTCTCCATCTCC-3′. In 3′-RACE, the adapt primer was 5′-GCGGAAATGGTCAGCCCTGTTGC-3′ and nested primer was 5′-CCAGCAGGGAGTGGTTGAGTTG-3′. RACE products were cloned using a TOPO zero blunt cloning kit (Invitrogen, Carlsbad, CA) and sequenced. To clone the entire cDNA, oligonucleotide primers annealing to the 5′ and 3′ end of the 5′-RACE and 3′-RACE product, respectively, were synthesized, followed by polymerase chain reaction (PCR) with the testis cDNA library as template. Primer sequences were 5′-GCCACCCATCCGAGGCCTTGTGGTT-3′ and 5′-GGGGAAGCAAGCAGGAGGCTACAGGTC-3′. The complete cDNA was cloned into TOPO zero blunt and transferred to a pcDNA3 expression vector and sequenced.

Northern blot analysis

Hybridization to multiple tissue Northern blot (Clontech) or to RNA Master blot (Clontech) was performed using Express-Hybridization solution (Clontech), according to the manufacturer's instructions. Briefly, a 1.5-kb *Eco*RI/*Eco*RI fragment of mouse BPI cDNA was labeled with ^{32}P-deoxy-cytidine 5′-triphosphate using the Rediprime™ II kit (Amersham Pharmacia Biotech, Little Chalfont, UK). High-stringency hybridization was performed at 68°C. The membrane was washed with 2× saline sodium citrate (SSC), 0.05% sodium dodecyl sulfate (SDS), for 3 × 10 min at room temperature and 0.1× SSC, 0.1% SDS, 2 × 20 min at 50°C. Membranes were analyzed using a Molecular Imaging FX analyzer (BioRad, Hercules, CA).

Reverse transcription (RT)-real-time PCR

RNA was extracted from mononuclear bone marrow cells and cell lines using Trizol LS reagent (Invitrogen). RT of 2 μg total RNA was performed using the Taqman kit (Applied Biosystems, Foster City, CA) and oligo-dT primer. RT real-time-PCR was performed with Taqman probes and the 7000 sequence detection system (Applied Biosystems), according to the manufacturer's instructions. Specific primers and probe for mouse BPI were ordered from Applied Biosystems with mouse BPI cDNA as template. The BPI primer sequences were: forward primer 5′AGATCAAGCACCTGGGAAAGG 3′, reverse 5′ GCATCTCGATCTTGGGATTAGGAAT, and probe 5′ CCATGCTGTAGAATTC 3′. For analysis of human BPI mRNA, primer and probe specific for human BPI were used (Applied Biosystems, #Hs00175186_m1). In RT real-time-PCR, 40 ng cDNA was used as a template. RT real-time-PCR of β2-microglobulin (Applied Biosystems, #4326319E) was used as an internal control for equal loading. Data were collected and analyzed with the Sequence Detector v.1.1 software (Applied Biosystems). Relative quantitative data in induction experiments (see Figs. 5 and 6) were calculated based on the $\Delta\Delta C_T$ method: normalization, $\Delta C_T = C_T$ (sample) – C_T (β2-microglobulin); $\Delta\Delta C_T = \Delta C_T$ (sample-induced) – ΔC_T (sample-uninduced); relative quantification = $2^{-\Delta\Delta CT}$ (reviewed in ref. [21]). In an analogous manner, relative quantification of mRNA levels in testis and bone marrow (see Fig. 4) was calculated based on ΔC_T [bone marrow–ΔC_T (testis); relative quantification=$2^{-(\Delta CT\ \text{bone marrow}-\Delta CT\ \text{testis})}$].

Production of polyclonal antiserum

Rabbit antiserum production was performed by Innovagen (Lund, Sweden). Briefly, the amino acid sequence CNGKWMSRKNFLKAGG from the predicted BPI sequence was selected as a result of high-surface exposure and antigenic probability. The corresponding polypeptide was synthesized with a keyhole limpet hemocyanin carrier, followed by repeated immunizations of a rabbit. Preimmune serum was collected prior to immunization. The immunoreactivity against the peptide was verified with enzyme-linked immunosorbent assay.

Immunocytochemistry

Cytospin preparations were fixed in 4% formaldehyde in 0.1 mol/l phosphate buffer, pH 7.4, for 20 min at room temperature. Cell permeabilization was performed by incubation in PBS containing 1% Triton X-100 at room temperature for 30 min. Unspecific binding was blocked with PBS containing 1% bovine serum albumin (BSA; Sigma Chemical Co.) for 10 min. Antiserum or preimmuneserum was diluted in PBS containing 0.25% BSA, and binding of primary antibody was performed by incubating at room temperature for 1 h. After washing with PBS, the preparations were incubated at room temperature for 1 h with alkaline phosphatase-conjugated, secondary antibody (D0306, Dako Cytomation, Denmark), diluted 50 times in PBS containing 0.25% BSA. Enzymatic reaction with Fast Red TR/Naphthol AS-MX was performed according to the manufacturer's instructions (Sigma Chemical Co.). The preparations were counterstained with Mayer's hematoxylin and mounted.

Assay for bactericidal activity

Linearized pcDNA3 (control) or pcDNA3, including cDNA encoding human BPI or mouse BPI, was transfected into HEK293 cells by electroporation (0.2 kV, 960 μF). Polyclonal stable-transfected cell lines were established by selection by continuous growth in the presence of geneticin (0.8 mg/ml). For determination of antibacterial activity, HEK293 cells were seeded in medium containing 1% FCS, and conditioned medium was collected after 3 days. The conditioned medium was tenfold, concentrated using Viva spin 2 columns (Vivascience, Hannover, Germany). Exponentially growing *J5 E. coli* (ATCC) were washed with PBS and 30,000 colony-forming units (cfu) in 100 μl PBS, mixed with varying volumes of conditioned medium, as indicated, in a total volume of 130 μl. After incubation in room temperature for 90 min, the mixtures were diluted 20-fold with PBS, and 40 μl dilution was plated on SOB agar plates for determination of the number of remaining bacterial cfu.

RESULTS

Cloning of mouse BPI cDNA

A Blast search of the public databases among mouse cDNAs for sequence similarities to human BPI cDNA indicated the mouse EST from epididymis tissue (#BB584418). The sequence (676 bp) showed a high similarity to human BPI cDNA and also contained an open reading frame (ORF) encoding an amino acid sequence with high similarity to parts of human BPI. This finding prompted us to clone the entire mouse cDNA. To this end, 5′- and 3′-RACE primers were designed and used in RACE reactions using a cDNA library from mouse testis, as described in Materials and Methods.

The cloned cDNA of 1631 bp contains an ORF encoding a 54-kDa protein, including a putative signal sequence (**Fig. 1**). The predicted molecular weight was confirmed by in vitro translation (data not shown). The putative ATG translation start codon is flanked by a Kozak consensus translation initiation site sequence [22]. Upon comparison of the predicted amino acid sequence with that of human BPI, striking similarities are found (Fig. 1). Overall amino acid identity is 53%, and similarity is 71% (**Table 1**). The amino acid sequence identity between murine BPI and murine LBP is significantly lower (37%), underlining the close relationship between murine and human BPI. The predicted isoelectric point of the mouse protein is 9.1, and that of human BPI is 9.5. To see whether the mouse protein sequence meets the requirements of folding into a three-dimensional (3D) structure, similar to that of human BPI [23], a 3D simulation was performed using Deepview/Swiss-PdbViewer, GlaxoSmithKline, http://www.expasy.org/spdbv/. The simulation predicted almost identical 3D structures for the mouse and human BPI (**Fig. 2**). We conclude that the cloned cDNA encodes a mouse ortholog to human BPI.

TABLE 1. Amino Acid Sequence Identity and Similarity of Murine and Human BPI.

	Identity (%)	Identity or similarity (%)
Murine BPI/human BPI	53	71
Murine BPI/murine LBP	37	57
Human BPI/human LBP	43	61
Murine BPI/human LBP	37	57
Murine LBP/human LBP	66	80

The mouse BPI-cDNA sequence has been deposited in GenBank, accession number AY853179. It has been scanned against mouse EST in GenBank, and apart from the above-mentioned BB584418, scanning identified BB073453, which showed significant homology. Both of the identified sequences were from epididymis of adult male *Mus musculus*.

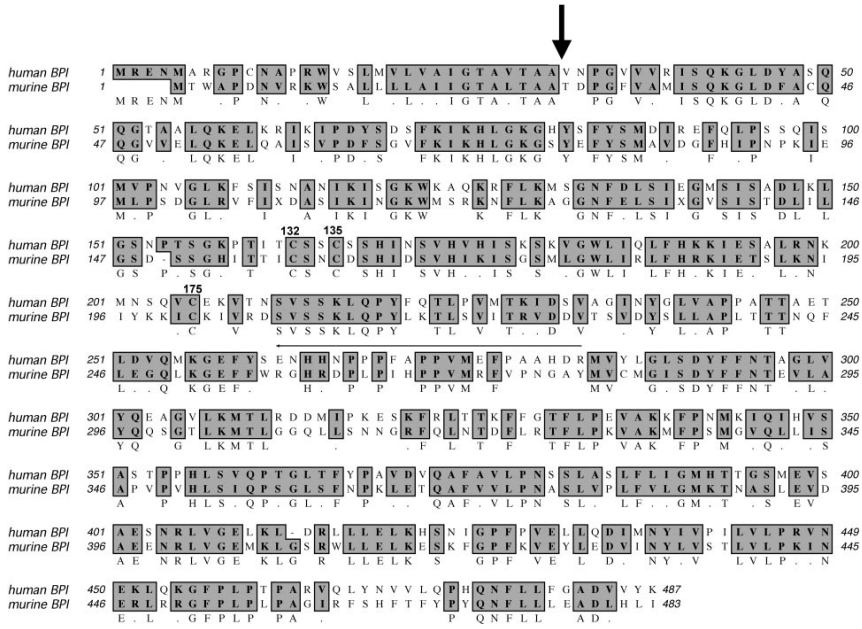

Fig. 1. Alignment of amino acid sequence of human and mouse BPI. Identical or conserved residues within boxes. The numbering of the conserved cysteines starts at the beginning of mature human BPI, indicated by the arrow. The proline-rich linker is indicated with a line.

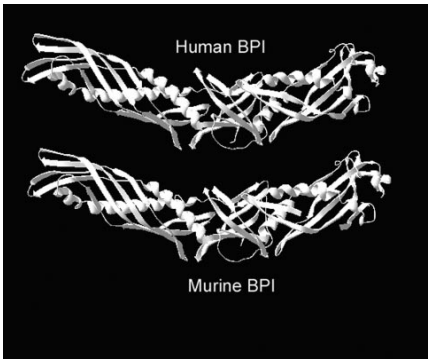

Fig. 2. 3D simulation of the structure of mouse BPI. Using the 3D structure of human BPI [23] as template, a 3D simulation was performed, using Deepview/Swiss-PdbViewer, GlaxoSmithKline, http://www.expasy.org/spdbv/.

Gene structure analysis

A Blast search of mouse BPI cDNA to the mouse genome in the National Center for Biotechnology Information Entrez Genome database showed that BPI is located on chromosome 2 at positions 158.8–158.9 Mb. Mouse BPI cDNA corresponded to the predicted UniGene cluster Mm.260883 and the hypothetical protein 9230105K17. The alignments of the cDNA to genomic sequence revealed 15 exons organized in a manner close to that of human BPI on chromosome 20 [24]. Moreover, the gene for mouse BPI on chromosome 2 is located close to the gene for LBP. This is similar to the organization of the corresponding human genes on chromosome 20 [24, 25]

Mouse BPI is expressed in testis and epididymis

As described above, the mouse BPI cDNA was cloned from a testis cDNA library. To investigate its tissue-expression pattern, a multi tissue Northern blot analysis was performed using the cloned cDNA as a probe. Of all the tissues analyzed, testis was the only one that produced a strong signal (**Fig. 3A**). The transcript size of approximately 1.7 kb was in good agreement with the 1631-bp cloned cDNA, indicating that the cloned sequence constitutes full-length cDNA. To extend the analysis to a broader number of tissues, an RNA Master blot analysis was performed. Once again, a strong signal was detected from testis but also from epididymis, a tissue not present on the previous Northern blot membrane (Fig. 3B). To verify that mouse BPI was expressed in testis tissue, we analyzed expression in the 15P-1 and TM4 cell lines derived from mouse testis. 15P-1 and TM4 show a Sertoli-like phenotype [18, 19] and have previously been reported to express antimicrobial peptides [26]. By RT-PCR, expression of mouse BPI was identified in both cell lines. The level of expression was comparable between the two cell types, as determined by gel analysis of PCR products (data not shown). Expression was corroborated by RT real-time-PCR analysis, showing mean Ct values of 34.1 (TM4) and 36.8 (15P-1). By Southern blot analysis and sequencing of PCR products, the identity of the PCR product as mouse BPI was verified (data not shown). The finding of BPI expression in murine testis cells led us to investigate the possible expression of BPI in human testis. To that end, BPI-mRNA levels in mouse and human testis RNA from primary tissue were analyzed with RT real-time-PCR. Mouse and human testis RNA were from BD Bioscience (San Jose, CA)/Clontech, #64045-1 and #64101-1, respectively. The efficiency of the PCRs of mouse and human BPI cDNA were compared by amplification of different amounts of pcDNA3 containing mouse and human BPI, respectively. Amplification of the same amount of human and mouse BPI cDNA resulted in comparable Ct values (data not shown), thus indicating that results of human and mouse real-time PCR analyses are quantitatively comparable. This way, mouse and human testis showed robust BPI-mRNA expression, but BPI expression is higher in mouse testis as compared with that of human testis (mean Ct values of 26.0 and 28.7, respectively; **Fig. 4**).

Expression of mouse BPI mRNA in bone marrow and induction of expression with ATRA

The neutrophil is the major storage compartment of human BPI, synthesized in precursor cells in the bone marrow [2, 3]. RNA from bone marrow tissue was not present on the blotting membranes described above. Therefore, RNA from mouse mononuclear bone marrow cells was also analyzed for BPI expression by RT real-time-PCR. Expression of BPI was observed with a mean Ct value of 34.9. Corresponding analyses of

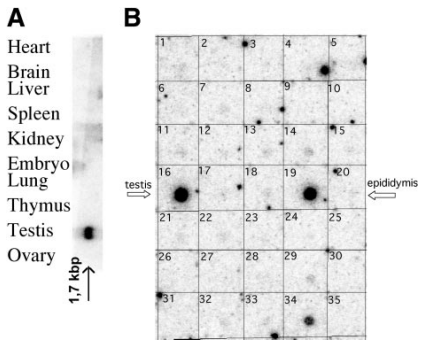

Fig. 3. Tissue-expression pattern of mouse BPI. (A) Hybridization to mouse multitissue Northern blot (Clonetech) or to (B) RNA Master blot (Clonetech), using mouse BPI cDNA as probe was performed as described in Materials and Methods. The tissues of the Master blot were: 1, brain; 2, eye; 3, liver; 4, lung; 5, kidney; 6, heart; 7, skeletal muscle; 8, smooth muscle; 9 and 10, blank; 11, pancreas; 12, thyroid; 13, thymus; 14, submaxim gland; 15, spleen; 16, testis; 17, ovary; 18, prostate; 19, epididymis; 20, uterus; 21, embryo day 7; 22, embryo day 11; 23, embryo day 15; 24, embryo day 17; 25, blank; 26, yeast RNA; 27, yeast tRNA; 28 and 29, *E. coli* RNA; 30, blank; 31, poly r(A); 32, Cot1DNA; 33 and 34, mouse DNA; 35, blank. Hybridization to a 1.5-kb transcript from testis (A) and centrally positioned hybridization to RNA from testis and epididymis (B) are indicated with arrows. The randomly off-center-located dots represent unspecific, artifactual hybridization, which was repeated with identical results, except for the unspecific hybridization, which changed position between hybridization.

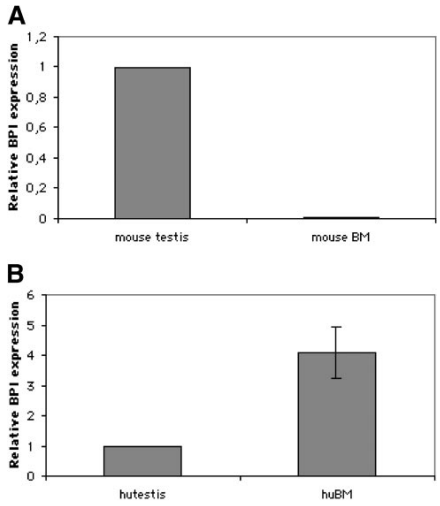

Fig. 4. Tissue-expression pattern of mouse (A) and of human (B) BPI mRNA in testis and bone marrow. RNA extracted from bone marrow cells and RNA from testis tissue were analyzed for BPI-mRNA expression by RT real-time-PCR. The relative expression levels were calculated according to the $\Delta\Delta C_T$ method as described in Materials and Methods. Shown are the relative levels of expression in bone marrow (BM) as normalized to that of testis. Mean Ct values are: mouse testis, 26.0; mouse BM, 34.9; human testis (hutestis), 28.7; human BM (huBM), 25.1. Bar = SEM, n = 3.

human BPI expression in mononuclear bone marrow cells resulted in a mean Ct value of 25.1, suggesting that BPI expression is several hundred-fold higher in human bone marrow, as compared with mouse bone marrow (Fig. 4). To corroborate bone marrow expression of mouse BPI, we analyzed the promyelocytic MPRO cell line using RT real-time-PCR analysis. MPRO cells are blocked at the promyelocytic stage of differentiation, but induction with ATRA results in terminal neutrophil differentiation including expression of secondary granule proteins such as lactoferrin and neutrophil gelatinase [20, 27]. Promyelocytic mouse MPRO cells showed expression of BPI with a mean Ct value of 33.4. The expression was enhanced several-fold by induction with ATRA in a dose-dependent manner (**Fig. 5A**). The response to ATRA could be discerned after 2 h and was further increased with longer incubation with ATRA, reaching a maximum after 12 h of ATRA induction (Fig. 5B). To investigate whether ATRA induction of BPI expression was dependent on de novo protein synthesis, we performed induction in the presence of 100 μg/ml cycloheximide. More than 95% of the total protein synthesis was inhibited by cycloheximide, as determined by biosynthetic labeling and trichloric acetic acid precipitation. The ATRA-induced BPI expression was insensitive to cycloheximide (Fig. 5C), suggesting that direct effects of the receptor for ATRA on the BPI gene are involved. As with MPRO cells, ATRA also increased the expression of BPI in Sertoli cells; treatment of 15P-1 cells with ATRA increased expression of BPI almost tenfold (**Fig. 6**). Moreover, ATRA induction of BPI in 15P-1 cells showed kinetics similar to that in MPRO cells (data not shown). The optimal response of 15P-1 cells occurs at 1 μM ATRA, and MPRO cells respond to even higher concentrations (10 μM) of ATRA (compare Figs. 5A and 6). One possible explanation for this difference in dose response between the cell lines might be the presence of mutated receptor for ATRA in MPRO cells, acting in a dominant-negative manner, thus resistant to high concentrations of ATRA [20].

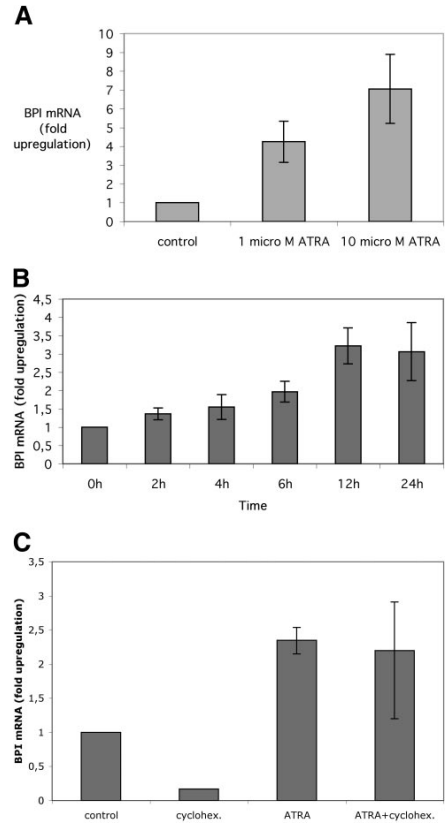

Fig. 5. ATRA induces expression of BPI mRNA in MPRO cells. RNA was extracted, and mRNA expression was determined by RT real-time-PCR analysis. The relative expression levels were calculated according to the $\Delta\Delta C_T$ method as described in Materials and Methods. (A) Dose-response induction of BPI mRNA in MPRO cells treated with 1 μM or 10 μM ATRA for 24 h. (B) Time kinetics for induction of BPI mRNA in MPRO cells treated with 10 μM ATRA for up to 24 h. (C) Induction of BPI mRNA in the absence of de novo protein synthesis; MPRO cells treated with 10 μM ATRA for 6 h in the presence or absence of 100 μg/ml cycloheximide. Bars = SEM, n = 3.

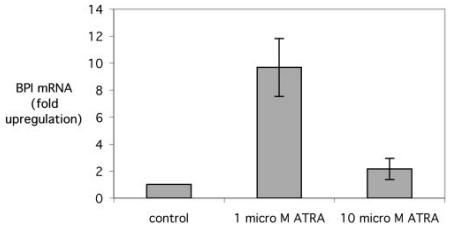

Fig. 6. ATRA induces expression of BPI mRNA in 15P-1 cells, which were treated with 1 μM or 10 μM ATRA for 24 h. RNA was extracted, and mRNA expression was determined by RT real-time-PCR analysis. The relative expression levels were calculated according to the $\Delta\Delta C_T$ method as described in Materials and Methods. Bars = SEM, n = 3.

BPI protein in cytoplasmic granules of bone marrow neutrophils

To identify the BPI protein using immunohistochemistry, we raised a polyclonal antiserum as described in Materials and Methods. Although the number of neutrophils in mouse peripheral blood is low, neutrophils and neutrophil precursors constitute ~40% of the mouse bone marrow [28], making the bone marrow an excellent source of progenitors and mature neutrophils. Mouse bone marrow cells were collected, cytospin preparations were prepared, and immunocytochemistry was performed as described. As shown in **Figure 7A**, a strong reactivity was obtained with immune serum, and preimmune serum resulted in background staining only (Fig. 7B). The labeling showed a granular pattern in the cytosol of mature neutrophils, indicating storage of BPI in cytoplasmic granules (Fig. 7A).

Mouse BPI possesses antibacterial activity

A major biological activity ascribed to human BPI is its cytostatic and cytotoxic effect upon Gram-negative bacteria (reviewed in ref. [29]). We therefore investigated whether mouse BPI protein also possesses antibacterial activity. For this purpose, cDNA of mouse or human BPI was cloned into pcDNA3 and stably expressed in HEK293 cells. By RT real-time-PCR analysis, the expression of human and mouse BPI in HEK293 cells was verified, with comparable Ct values (data not shown). As HEK293 cells lack storage granules, BPI is expected to be extracellularly secreted from these cells. Conditioned medium was analyzed for antibacterial activity toward the *E. coli* strain J5, as described in Materials and Methods. Antibacterial activity was present in conditioned medium, showing a cytotoxic/cytostatic effect on *E. coli* (**Fig. 8**), indicating that BPI with antibacterial activity was indeed secreted from the cells. The antibacterial activity of mouse BPI was of a magnitude similar to that of human BPI, suggesting comparable activities against *E. coli*.

DISCUSSION

BPI has been reported to be present in human, bovine, and rabbit neutrophils but not in mice [11]. However, we report here the cloning of a mouse ortholog to human BPI, as determined from its structural and functional characteristics. Mouse BPI was found to be expressed in testis, epididymis, and myeloid cells of the bone marrow.

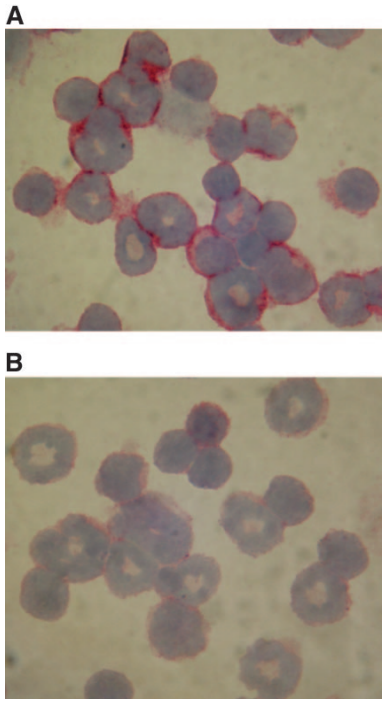

Fig. 7. Cytoplasmic granular localization of BPI in mouse bone marrow granulocytes. BPI in mouse bone marrow cells were identified by immunocytochemistry as described in Materials and Methods. (A) Immune serum; (B) preimmune serum.

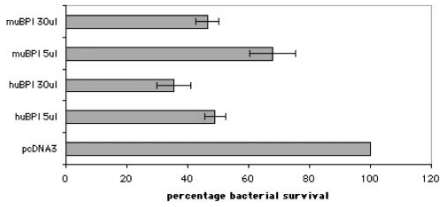

Fig. 8. Mouse BPI shows bactericidal activity against *E. coli*. HEK293 cells were stably transfected with mouse or human BPI (muBPI or huBPI) or with pcDNA3 as negative control. Varying amounts of concentrated HEK293 supernatants were incubated with the *E. coli* strain J5 as described in Materials and Methods. Remaining cfu after incubation, as normalized to the value obtained with negative control, are shown. Bars = SEM, n = 4.

Other BPI family members are expressed in mouse epithelia (reviewed in ref. [12]). Recently, the BPI family was expanded to include RY (also designated LPLUNC2-4) orthologs, expressed mainly in human and mouse olfactory epithelium [14, 15], as well as mouse RYSR (LPLUNC2), expressed in the testis [14]. None of the BPI relatives in mouse, however, show more than 30% sequence identity to human BPI [14, 17]. Mouse BPI shows 53% amino acid identity to the human form, which is thus higher than any other human or mouse BPI family member. Moreover, human and mouse BPI share conserved genomic features, as determined by exon organization and their localization close to the gene for LBP in both species.

The sequence similarity between human and mouse BPI is higher than that between human BPI and human LBP, a plasma protein closely related to BPI [24], or mouse BPI and mouse LBP, further emphasizing the high degree of conservation between human and mouse BPI. Sequence identity and similarity between mouse and human BPI and LBP are summarized in Table 1. Conserved motifs between human and mouse BPI include a proline-rich linker region, which connects the amino- and carboxy-terminal domains [23]. Moreover, cysteine residues involved in intramolecular disulfide bonds are conserved. These include cysteines 135 and 175 (numeration from the amino-terminal of mature, human BPI), which form a disulfide bond necessary for the correct folding of human BPI [23, 30]. Disruption of this bond dramatically decreased the LPS binding and bacteria toxicity [30, 31]. In addition, cysteine 132, important for dimer formation of human BPI [31], is conserved between the two BPI orthologs. From the sequence similarities, it can be predicted that mouse BPI adopts a spatial structure similar to that of the human protein. Indeed, computer modeling indicated an almost identical 3D structure for mouse BPI and human BPI. Given the similar structure, it is not surprising that human and mouse BPI share antimicrobial activity against the *E. coli* strain J5, as shown from overexpression data in HEK293 cells. Secretion of BPI into the supernatant may be related to a lack of efficient storage in HEK293 cells. Thus, structural and functional characteristics support a close relationship between human and mouse BPI. Taken together, we can conclude from the present data that the cloned cDNA encodes a true mouse BPI ortholog.

Human BPI is synthesized in promyelocytes of the bone marrow [2, 32] and in mucosal epithelial and dermal fibroblasts upon appropriate stimulation [4, 5]. Our present data indicate that mouse BPI is expressed in myeloid bone marrow cells and in cells of testis and epididymis. A differentiation-related expression of mouse BPI in myeloid cells was indicated by our demonstration that expression of BPI in promyelocytic MPRO cells was enhanced six- to eightfold by treatment of the cells with ATRA, which also induces differentiation of these cells. Thus, mouse BPI appears to be developmentally expressed in myeloid cells during the promyelocyte stage, as is the case in human myeloid cell differentiation. Consistent with this concept, mature neutrophils of mouse bone marrow showed granular immunocytochemical staining for BPI, indicating that the protein was stored in cytoplasmic granules. Taken together, the present RT-PCR analyses of bone marrow and promyelocytic MPRO cells, as well as protein identification in mature neutrophils, clearly demonstrate BPI to be present in mouse neutrophils. However, as judged by real-time PCR analysis of mRNA, murine neutrophils seem to produce lower amounts of BPI as compared with human neutrophils. However, in the absence of tools for quantitative protein analysis, the absolute levels of mouse BPI protein in neutrophils cannot be determined.

Results from RNA hybridization and RT real-time-PCR analyses on testis RNA preparations indicated that mouse BPI is constitutively expressed in testis and epididymis. The absolute expression level is, from present data, difficult to estimate, but strong signals from the RNA hybridization analyses suggest a robust expression. The finding that two Sertoli cell lines express BPI argues strongly against that contaminating neutrophils are responsible for the BPI signal in RNA preparations but instead, indicate that Sertoli epithelial cells produce BPI in the testis. Moreover, our results also show an expression of human BPI in testis at a level almost as high as in mice. This is in agreement with the finding that an EST from human testis (GenBank #AA421088) does show high identity to human BPI. The tissue-expression pattern in bone marrow and testis of mouse and human BPI seems different; mice express a higher amount of BPI in testis than in bone marrow, whereas the reverse relationship is found in human tissues (Fig. 4). The relatively low levels of BPI mRNA in mouse bone marrow provide an explanation why previous attempts to identify BPI-like species in mouse myeloid cells were unsuccessful.

The observed ATRA-induced expression of mouse BPI in promyelocytic MPRO cells and in 15P-1 Sertoli cells indicates a mechanism for BPI induction, which is common between tissues. The mechanisms for ATRA-induced differentiation of human myeloid cells include an increase in the transcription factor C/EBPε, which contains ATRA-response elements in its promoter [33]. No information on functional regulatory elements of the mouse BPI gene is available, but a role of C/EBPs in the positive regulation of the human BPI gene [34] is consistent with indirect ATRA effects mediated via C/EBPε. However, although no ATRA-response elements have been defined, the present finding that ATRA-induced expression of mouse BPI is at least partly independent of de novo protein synthesis indicates direct effects of ATRA. Therefore, ATRA might exert indirect and direct effects on BPI expression.

The functions of most of the BPI family members are believed to be related to antimicrobial effects in the innate immune system. The storage of BPI in mouse and human neutrophils and common antibacterial effects in vitro support the concept that both BPI orthologs are important for the neutrophil-mediated defense against Gram-negative bacteria. What is the functional role of BPI in testis and epididymis? In addition to BPI-related proteins, other antimicrobial proteins such as cysteine-rich secretory protein 3, cryptdin, human cationic antimicrobial protein-18, human epididymis 2, and defensins are expressed in these tissues [26, 35–39], several of which are also present in leukocytes. It is therefore possible that mouse BPI in testis and epididymis has antimicrobial functions, such as a role in antibacterial defense during fertilization, as previously suggested for other polypeptides [36, 40]. As an antibacterial agent, BPI acts in synergy with defensins and cathelicidins on Gram-negative bacteria [41]. Coexpression of several antibacterial peptides may therefore be neces-

sary for required protection against bacterial invasion. Recently, it was reported that β-defensin plays an important role in induction of sperm motility by inducing Ca^{2+} uptake [42]. Hence, antimicrobial proteins can have other specific properties when expressed in various tissues. Indeed, BPI has been implicated in biological functions besides those related directly to bactericidal effects, e.g., to act as an endogenous inhibitor of angiogenesis [43], to neutralize the inflammatory effects of LPS [44], or to stimulate phagocytosis by complement activation [45]. The present identification of mouse BPI opens the possibility to investigate its physiological and pathophysiological role in vivo by elimination of the gene in transgenic mice.

In conclusion, we report the identification of a mouse ortholog to human BPI, which is expressed in testis, epididymis, and bone marrow. BPI protein is stored in neutrophil cytoplasmic granules. Mouse BPI shows high structural similarity to human BPI, with which it shares antibiotic effects against Gram-negative bacteria.

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