Isolation, Characterization, and Antimicrobial Properties of Bovine Oligosaccharide-binding Protein

A MICROBICIDAL GRANULE PROTEIN OF EOSINOPHILS AND NEUTROPHILS*

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Peptidoglycan recognition proteins (PGRPs) constitute a recently characterized family of pattern-recognition molecules that are conserved from insects to humans and are implicated in mammalian innate immunity. Here we report the isolation, characterization, cDNA cloning, and antimicrobial activities of a bovine PGRP ortholog termed bovine oligosaccharide-binding protein (bOBP). Milligram quantities of bOBP were purified from peripheral leukocytes, thus allowing for the characterization of the disulfide array and for determining the in vitro antimicrobial activities of the native protein. Of the tissues analyzed, bOBP mRNA was detected only in bone marrow where the protein is synthesized as a 190 amino acid precursor. The mature 169 amino acid protein is stored in the cytoplasmic granules of neutrophils and eosinophils but is absent from lymphocytes, monocytes, and platelets. bOBP was microbicidal for Gram-positive and Gram-negative bacteria and yeast at low micromolar concentrations. The finding that bOBP was microbicidal for organisms in which peptidoglycan is absent (Cryptococcus neoformans) or buried (Salmonella typhimurium) indicates that previous conclusions about the specificity of peptidoglycan recognition proteins must be reevaluated and suggests that other envelope components may mediate the antimicrobial action of PGRP family members.

Studies over the past two decades have revealed that antimicrobial innate immunity plays a critical role in host defense mediated in part by pattern-recognition proteins and microbicidal effector molecules that contain infection prior to an adaptive immune response (1). Pattern-recognition proteins distinguish invading microbes by identifying conserved microbial structures that are not expressed by host cells (2). Microbial killing is mediated by granulocytes and macrophages that produce oxygen- and nitrogen-derived toxins (3, 4) as well as microbicidal proteins and peptides (1).

Among the pattern-recognition proteins of innate immunity are lipopolysaccharide (LPS)1-binding protein and bactericidal/permeability-increasing protein, molecules that bind LPS by amino-terminal structures conserved between the two proteins (5, 6). Bactericidal/permeability-increasing protein is potently bactericidal for Gram-negative bacteria (7, 8) and neutralizes the inflammatory properties of LPS (9). In contrast, LPS-binding protein is not bactericidal and activates an inflammatory response in myeloid cells upon LPS binding (10, 11). Toll-like receptors (TLRs) conserved from insects to humans (12) are key elements of innate immunity (13, 14). More than ten mammalian TLRs have been identified, each of which may have pattern-recognition functions (15). For example, TLR2 recognizes Gram-positive bacteria by binding peptidoglycan or lipoteichoic acid (15, 16), TLR4 identifies Gram-negative bacteria through LPS interactions (15, 17, 18), TLR5 recognizes flagellin (15, 19), and TLR9 identifies bacterial (CpG)DNA (15, 20). TLR-ligand complexes signal the induction of inflammatory cytokines and chemokines.

Mannose-binding protein is a soluble pattern-recognition protein found in the serum of all mammals studied (21). A collectin, mannose-binding protein activates complement (21) and opsonizes microbes (22) by binding to carbohydrate moieties of bacterial and fungal cell envelopes. Humans deficient in circulating mannos-binding protein demonstrate increased vulnerability to a number of microbial pathogens (21, 23).

Peptidoglycan recognition proteins (PGRPs) are recently characterized components of innate immunity, which are reported to bind specifically to peptidoglycan moieties. These studies were the basis for concluding that PGRPs selectively contribute to innate immunity by detecting Gram-positive bacteria (24, 25). Silkworm PGRP, the first member of the family, was implicated in the innate immune response by its ability to trigger the prophenoloxidase cascade in the presence of peptidoglycan or its glycan moiety (26, 27). Orthologous proteins have been found in other lepidopteran insects (28–30) and in Drosophila (31) wherein PGRP gene expression is up-regulated in larvae inoculated with Gram-negative bacteria (28, 31). Mammalian PGRPs have been reported in humans (24, 26, 28), mice (26, 28), rats (GenBank™ accession number AF154114), and camels (GenBank™ accession number AF154114).

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The abbreviations used are: LPS, lipopolysaccharide; PGRP, peptidoglycan recognition protein; TLR, toll-like receptor; bOBP, bovine oligosaccharide binding protein; BD-13, BOP derived peptide-13; RP-HPLC, reversed-phase high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Fnoc, N-(9-fluorenyl)methoxycarbonyl; RACE, rapid amplification of cDNA ends; PIPES, 1,4-piperazinediethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone or tosylphenylalanyl chloromethyl ketone.
AJ131676). PGRP mRNAs have been localized to human and mouse bone marrow and polymorphonuclear leukocytes (26, 28) but not to lymphocytes or monocytes (26). Genomic analysis has determined that there are four human genes (24) and at least eight Drosophila genes (31) that have significant PGRP homology. Three of the human genes and three of the Drosophila genes predict products with transmembrane domains (24, 31).

In this study, we isolated several milligrams of a bovine PGRP, which we have termed oligosaccharide-binding protein because it was found to kill microorganisms in a peptidoglycan-independent manner. The precursor and mature protein structures, including the conformation of the tri-disulfide array, were determined. Furthermore, tissue expression analysis and immunolocalization studies were performed to ascertain the sites of biosynthesis and the storage of bOBP.

**EXPERIMENTAL PROCEDURES**

**Bovine Granulocytes**—Granulocytes were purified from fresh citrated bovine blood (dairy cattle) as described previously (32). Preparations contained an average of $1 \times 10^{10}$ cells/liter of whole blood of which $93 \pm 3\%$ were neutrophils and $4 \pm 1\%$ were eosinophils. Some granulocyte preparations were treated with 2 mM diisopropylfluorophosphate (33) or protease inhibitor mixture tablets (Complete™ EDTA-free, Roche Molecular Biochemicals) prior to protein extraction. Eosinophil-enriched populations of granulocytes were obtained by centrifugation over a Percoll continuous density gradient prepared according to the manufacturer’s directions (Amersham Biosciences). $4 \times 10^9$ bovine granulocytes suspended in Hanks’ balanced salt solution plus Ca$^{2+}$ and Mg$^{2+}$ were layered over the gradient and centrifuged at 7500 x g for 10 min, and the supernatant was collected. Subsequent quantification of bOBP was determined spectrophotometrically at 280 nm using the glutaraldehyde method as described previously (37). The BDP-13-ovalbumin conjugate was suspended in phosphate-buffered saline and used to immunize two New Zealand White rabbits (Zymed Laboratories Inc., South San Francisco, CA). Serum samples were collected after 10 weeks when the specific antibody titer, as determined by enzyme-linked immunosorbent assay, was $\sim 1:5000$. IgG was prepared from antiserum by DEAE-Econo-Pac chromatography (Bio-Rad) per the manufacturer’s instructions.

**Isolation and Characterization of bOBP**—Acid extracts of $1 \times 10^5$ cells equivalents of bovine leukocytes or granule preparations were loaded onto a $10 \times 25$ cm Delta Pak RP-HPLC C18 cartridge equilibrated in 0.1% trifluoroacetic acid (solvent A) at a flow rate of 10 ml/min. A linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (solvent B) was applied to 2% min from 0 to 37%, 0.1% min from 37 to 38%, and 0.167%/min from 38 to 40%. 10-mL fractions were collected, lyophilized, and resuspended in 0.01% acetic acid. Samples containing bOBP were identified by Dot Blot analysis (as described below) using anti-bOBP IgG (as described above), and pure bOBP was obtained following a second round of RP-HPLC. Purity was confirmed by SDS-PAGE, acridine UREA, PAGE, and analytical RP-HPLC. Quantitation and composition analysis of bOBP was determined by amino acid analysis performed on samples hydrolyzed at 150°C for 2 h using the Aminoacid Analyzer (Waters, Milford, MA) according to the manufacturer’s directions. Subsequent quantification of bOBP was determined spectrophotometrically (1 mg/ml = 1.28 A$_{280}$). Purified protein was lyophilized, dissolved in 0.01% acetic acid at 2 mg/ml, and stored at $-70^\circ$C.

bOBP and trypsin digestion fragments of bOBP (see below) were subjected to size exclusion chromatography on a Bio-Gel P-60 column as described previously (32). Low molecular mass components (<10 kDa) identified by SDS-PAGE were further resolved by C$_{18}$ reversed-phase high liquid chromatography (RP)-HPLC, and fractions with antimicrobial activity were collected as described previously (32). BDP-13 among the low molecular weight antimicrobial peptides obtained was characterized by automated Edman sequencing on an Applied Biosystems 475A instrument configured with on-line phenylthiohydantoin amino acid analysis (32). The primary sequence was confirmed by comparison with the sequence predicted from the corresponding cDNA (see below) and by biochemical comparison with a synthetic congener (see below). Purified peptide was lyophilized, dissolved in 0.01% acetic acid at 100–500 μg/μl, and stored at $-20^\circ$C.

**Peptide Synthesis and Antibody Production**—BDP-13 was synthesized with a Milligen 9050 peptide synthesizer as described previously [(35), (36)] on Fmoc-L-valine-polyethylene glycol-polystyrene resin (Millipore, Milford, MA). Side chain protecting groups were 2,2,5,7,8-penta-methylchro-man-6-sulfonyl for arginine, trityl for glutamine and histidine, tert-butoxy-carbonyl for lysine, and tert-butylicarbonyl for tyrosine. Isoleucine was double-coupled. Following cleavage and deprotection at 22°C for 4 h with reagent K (trifluoroacetic acid/thioanisole/ethanediol/thiophenol/water (82:5.5:2.5:5.5)), the peptide was purified by C$_{18}$-RP-HPLC water/acetonitrile gradients and quantified by amino acid analysis (32, 35). Purified synthetic BDP-13 was indistinguishable from natural BDP-13 by acid urea-PAGE, analytical RP-HPLC, and in antimicrobial assays.

*Synthetic BDP-13 (5 mg) was conjugated to 5 mg of chicken ovalbumin using the glutaraldehyde method as described previously (37). The BDP-13-ovalbumin conjugate was suspended in phosphate-buffered saline and used to immunize two New Zealand White rabbits (Zymed Laboratories Inc., South San Francisco, CA). Serum samples were collected after 10 weeks when the specific antibody titer, as determined by enzyme-linked immunosorbent assay, was $\sim 1:5000$. IgG was prepared from antiserum by DEAE-Econo-Pac chromatography (Bio-Rad) per the manufacturer’s instructions.*
spectrometer in the linear mode. Samples (3 pmol each) were mixed with equal volumes of -cyano-4-hydroxycinnamic acid matrix prior to analysis (38).

Partial amino acid sequence was obtained by automated Edman degradation of cyanogen bromide cleavage fragments of bOBP on a Hewlett Packard 1100 instrument (UCI Biotechnology Research Facility, Irvine, CA) (32). The sequencer cartridge loaded with 300 pmol of bOBP was soaked in a solution of 10% cyanogen bromide in 90% formic acid for 2 h at room temperature. The cartridge was then washed with water, and the eluted material was diluted to 5% formic acid, reapplied to the cartridge, and sequenced. The internal sequences were compared with the amino acid sequence deduced from the bOBP cDNA (as described below). Disulfide Connectivities—Two nanomoles of bOBP was digested for 2 h at 37 °C with 1 μg of TPCK-treated trypsin in 50 μl of 0.1 M pyridine acetate, pH 6.6. The reaction was terminated by acidification with trifluoroacetic acid. Half of the digest was directly analyzed by RP-HPLC on a 4.6 × 250-mm C18 column equilibrated in 0.1% trifluoroacetic acid (solvent A) at a flow rate of 1 ml/min. A linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (solvent B) was applied at 0.5%/min for 90 min, and 1-ml fractions were collected. The other half of the tryptic digest was lyophilized, dissolved in 20 μl of 6 M guanidine...
hydrochloride, 0.2 mM Tris-HCl, 2 mM NaEDTA, pH 8.2, purged with nitrogen, and incubated at 50 °C for 30 min. The sample was reduced with 390 µM dithiothreitol for 4.5 h at 50 °C and alkylated with 3-fold excess iodoacetamide for 10 min in the dark. Excess alkylating reagent was quenched with dithiothreitol. The reduced and alkylated tryptic digest was analyzed by RP-HPLC under the same conditions as the non-reduced digest. A comparison of the two chromatograms identified three disulfide-containing fragments in the untreated digest. These three fragments were characterized by MALDI-TOF MS and automated Edman degradation.

cDNA Cloning—Total RNA was isolated from bovine bone marrow by guanidium thiocyanate-phenol extraction (39). Total RNA (1 µg) was used to synthesize cDNA, and 3′-RACE was performed according to the manufacturer’s protocols (Invitrogen) using a degenerate gene-specific primer, 5′-CARGTCGGCCNCAYTAYMG-3′. PCR amplification was carried out using the following cycling parameters: 95 °C for 1 min; 55 °C for 1 min; and 72 °C for 1 min for 35 cycles. Subsequently, 1 µg of total RNA was subjected to CDNA cloning and 5′-RACE according to manufacturer’s directions with the gene-specific primer 5′-TGGGATGGGTTGGGTGGAAGACCGAGACGCCCTCACACGGCG-3′. PCR-amplified RACE products were subcloned and sequenced as described previously (37). Once the 5′- and 3′-ends of the BDP-13 cDNA were known, a PCR product corresponding to the full-length BDP-13 precursor sequence was generated and characterized by sequence analysis. Sequence data were analyzed using Geneworks 2.5.1 (IntelliGenetics, Mountain View, CA). Amino acid sequences were analyzed using BLAST and Swiss Protein data bases. Protein and peptide theoretical masses were calculated by the Expasy Peptide Mass program (40).

Western Blot and Dot Blot Analyses—Acid extracts of bovine leukocytes, extracts of leukocyte granules, and purified bOBP were lyophilized, boiled for 2 min in dithiothreitol-containing SDS-PAGE sample buffer, subjected to Tricine–SDS–PAGE (12.5% acrylamide), and transferred to 0.22-µm nitrocellulose membranes (MSI, Westborough, MA) using the semi-dry electroblotting technique (41). For Dot blot analyses, RP-HPLC fractions were subjected to rotoevaporation briefly to remove acetonitrile, and they were applied in 1-µl aliquots to 0.22-µm nitrocellulose membranes. Western blot and Dot blot membranes were incubated with 390 µM dithiothreitol, quenched with 3-fold excess iodoacetamide for 10 min in the dark. Excess alkylating reagent was quenched with dithiothreitol. Western blot and Dot blot membranes were incubated with 1:100,000 goat—anti-bOBP IgG (Fig. 3A). For Dot blot analyses, RP-HPLC fractions were subjected to rotoevaporation briefly to remove acetonitrile, and they were applied in 1-µl aliquots to 0.22-µm nitrocellulose membranes. Western blot and Dot blot membranes were incubated with 1:100,000 goat—anti-bOBP IgG preabsorbed with BDP-13. Immunoreactive bands or Dots were detected with 1:100,000 goat—anti-bOBP IgG for 60 min at room temperature, slides were developed by exposure to X-ray film or using digital imaging (Alpha Innotech, San Leandro, CA).

Immunohistochemistry—Slides of eosinophil-enriched leukocytes were prepared on a cytocentrifuge, fixed in 4% paraformaldehyde for 10 min at 4 °C, and stored at 4 °C in 70% ethanol. Slides were rehydrated for 5 min in 1× phosphate-buffered saline and treated with Fc receptor blocking reagent (InnoveX BioSciences, Richmond, CA) for 20 min and then with 1% normal goat serum for 20 min. Following incubation with 1:40 anti-bOBP IgG for 60 min at room temperature, slides were developed with 1:100,000 goat—anti-rabbit IgG conjugated to horseradish peroxidase using SuperSignal West Pico substrate (Pierce). Blots were developed by exposure of x-ray film or using digital imaging (Alpha Innotech, San Leandro, CA).

RESULTS

Isolation, Characterization, and Synthesis of BDP-13—Approximately 2 × 10⁸ cell equivalents of acid-solubilized granule protein was fractionated on a Bio-Gel P-60 column, and the antibacterial activity in pooled eluent fractions was evaluated by radial diffusion assay (32). One broad P-60 peak that contained neutrophil β-defensins (32) was further fractionated by RP-HPLC. BDP-13, later identified as the carboxyl-terminal tridecapeptide of bOBP, eluted on C₁₈ RP-HPLC as a single peak at 23 min (32). After a subsequent round of RP-HPLC under slightly different gradient conditions, pure BDP-13 was obtained which was homogeneous by analytical RP-HPLC and acid-urea PAGE (data not shown). Automated sequence analysis was performed providing unambiguous assignment of the primary sequence of BDP-13, YKIIQQWPHYRRV (Fig. 1, double-underlined sequence). As discussed below, the sequence was confirmed by comparison with the bOBP cDNA. Synthetic BDP-13 was used to produce anti-bOBP antibody (see “Experimental Procedures”).

bOBP cDNA—The full-length bOBP cDNA was 688 nucleotides in length and predicted a 190-amino acid precursor protein (Fig. 1). Within the precursor, the sequence of the first 21 residues was characteristic of a signal peptide (45, 46), and the predicted signal peptide cleavage site was confirmed by characterization of native bOBP (169 amino acids, see below). BDP-13 corresponded to the 13 carboxyl-terminal residues of bOBP.

A BLAST search of GenBank™ identified bOBP to be a member of the peptidoglycan recognition protein family of proteins. Eight proteins with greatest sequence similarity to bOBP included seven PGRPs and a butterfly molt protein (Fig. 2). Four cysteines are completely conserved, and six cysteines are conserved in the mammalian sequences. The identity of amino acid sequences to bOBP ranged from 37 to 74%.

Isolation and Characterization of bOBP—bOBP was purified from acid extracts of bovine granulocytes and of granule preparations by RP-HPLC fractionation and immunologic detection with anti-bOBP IgG (Fig. 3A). Purified bOBP was homogeneous by RP-HPLC (Fig. 3B), behaved as a single 19-kDa band on SDS-PAGE (Fig. 3C), and had a mass of 18779.6 by MALDI-TOF MS. The mass data are consistent with a polypeptide composed of the 169 carboxyl-terminal residues of the bOBP precursor with additional modifications including the formation of three disulfides and the cyclization of glutamine 22 to form pyroglutamic acid at the amino terminus of the mature protein (theoretical mass = 18755.3). Consistent with this structure, the amino terminus was blocked as revealed by automated sequence analysis. The sequence of a cyanogen-bromide digestion fragment was determined to be GNYMHRVPASALAAQSL (Fig. 1, single underline). This sequence was identical to bOBP residues 127–145 predicted by the cDNA. The predicted pI of mature bOBP is 9.38 (47).

The average yield of BDP-13 from 1 × 10⁸ bovine granulocytes (~400 µl of whole blood) was 2.9 mg. In contrast, no more than 2.125 µg of BDP-13 was detected in an equivalent number of granulocytes, indicating that the major form of the bOBP gene product in leukocytes is the 169 amino acid molecule.

bOBP Disulfide Motif—RP-HPLC chromatograms of native and reduced/alkylated tryptic digests of bOBP revealed three peptides that were modified by reduction and alkylation, indicating the presence of disulfide bonds. Each of the corresponding peptides obtained from the native digest was analyzed by MALDI-TOF MS and six steps of Edman sequencing (Table I). An analysis of the masses and sequences obtained allowed for unambiguous assignment of the primary sequence of each peptide (Table I). The resultant motif of 1 pairings in bOBP—shown in Fig. 4. The resultant motif of 1 pairings in bOBP—shown in Fig. 4. The resultant motif was consistent with the pattern reported for Bombyx mori PGRP that contains two disulfides, those corresponding to the 1–6 and 3–4 pairs in bOBP.

Immunolocalization of bOBP—Immunohistochemical staining of bovine peripheral blood leukocytes with rabbit anti-
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bOBP IgG demonstrated that bOBP is expressed in eosinophils and neutrophils. Immunoreactivity was strongest in eosinophils, slightly less in neutrophils, but absent from lymphocytes and monocytes (Fig. 5). Extracts of purified lymphocytes and monocytes and of purified platelets were negative for bOBP when subjected to Western blot analysis (data not shown). The immunostaining of bovine neutrophils and eosinophils was punctate and cytoplasmic, consistent with granular storage of bOBP in these cells.

Distribution of bOBP mRNA—Because bOBP was isolated from granulocytes of circulating bovine blood, the detection of 0.9-kb bOBP mRNA in bone marrow extracts was not unexpected (Fig. 6). Six other tissues including liver and spleen (highly populated by myeloid elements), small intestine, kidney, lung, and trachea were analyzed and found to contain no detectable bOBP mRNA (Fig. 6 and data not shown).

Antimicrobial Activities of BDP-13 and bOBP—The ability of bOBP and BDP-13 to inhibit the growth of microorganisms was determined in radial diffusion inhibition assays. Significant zones of inhibition were observed with 10 μg/ml bOBP against S. aureus 502a, L. monocytogenes EGD, and E. coli ML35 (Fig. 7). The fungus, C. neoformans 271a, was less sensitive to bOBP than bacteria, but an appreciable zone of clearing was apparent at 30 μg/ml (570 μg/ml). The diameters of clearing were dose-dependent, demonstrating log-linear relationships in the range tested typical of antimicrobial peptides and proteins. At concentrations of 3–100 μM, BDP-13 was inactive against bacteria and weakly inhibited growth of C. neoformans at 30 and 100 μM. Neither bOBP nor BDP-13 inhibited the growth of a second yeast, C. albicans 16820, at concentrations of up to 100 μM (data not shown).

Antimicrobial suspension assays were used to determine the microbicidal activities of BDP-13 and bOBP. A 2-h incubation with 1.3 μM (25 μg/ml) bOBP induced at least three logs of killing of S. typhimurium SH6497, a polymyxin-resistant strain, and of L. monocytogenes 967 (Fig. 8). S. aureus 502a and L. monocytogenes 10403s were killed by at least three logs by 2.7 μM bOBP (50 μg/ml), whereas equivalent killing of S. typhimurium GAlE required twice as much protein (Fig. 8). E. coli ML35, although inhibited in the diffusion assay (Fig. 7), was resistant to bOBP concentrations of up to 200 μg/ml (10.7 μM) (data not shown). BDP-13 killed more than two logs of C. neoformans 271a at 10.7 μM (200 μg/ml) (Fig. 8).

**DISCUSSION**

bOBP is a leukocyte-derived member of the PGRP gene family that is expressed at high levels in circulating neutrophils and eosinophils. The average yield of bOBP was ~7 mg/liter of adult bovine blood. The abundance of the protein and the relative ease of purification facilitated the structural and functional studies described herein.

bOBP is the first vertebrate PGRP for which the amino acid sequence (Fig. 2) and tri-disulfide structure (Fig. 4) have been determined. Mass spectroscopic analyses demonstrate that bOBP is not glycosylated and that the amino terminus is a pyroglutamyl residue. The cystine motif is consistent with that of the two-disulfide motif of B. mori PGRP (25). In all PGRPs, one disulfide of 1–6 bridges the amino and carboxyl ends of the chain, and a second disulfide of 3–4 forms a heptapeptide loop, the sequence of which is highly conserved particularly in mammalian PGRPs, introducing an additional degree of backbone constraint that is absent in the invertebrate orthologs.

An assignment of the PGRP nomenclature to this family of proteins was rationalized by studies indicating that natural PGRP from B mori bound to peptidoglycan (27) and that recombinant PGRP from Trichoplusia ni and from Mus musculus bound well to peptidoglycan and Gram-positive bacteria but poorly to Gram-negative bacteria (26, 28). However, our studies

| Table I Cystine-containing peptides isolated from bOBP^a |
|----------------------------------------------------------|
| Disulfide bond   | Amino acid sequence of fragment^b | Measured mass^c | Theoretical mass^d |
|-----------------|----------------------------------|-----------------|-------------------|
| Cys^46-Cys^46   | YYVSH                            | 2078.58         | 2078.34           |
| Cys^3-Cys^137   | AAQSLL                           | 2077.58         | 2076.36           |
| Cys^39-Cys^64   | GXDVG                            | 2854.73         | 2855.15           |

^a Cystine-containing fragments of trypsin-digested bOBP were identified by RP-HPLC of tryptic digests of native and reduced/alkylated sample.

^b Tryptic digest fragments as shown in Fig. 4.

^c Mass was determined by MALDI-TOF MS.

^d Theoretical mass was calculated by the ExPASy PeptideMass program (40).

![Fig. 4. Disulphide connectivities of bOBP.](http://www.jbc.org/)

**Fig. 5. Immunolocalization of bOBP in peripheral leukocytes.** An eosinophil-enriched population of bovine granulocytes was prepared for immunocytochemistry as described under “Experimental Procedures.” Panels A and B, cells were stained with anti-BDP-13 IgG (see “Experimental Procedures”). Panel C, cells were stained with anti-bOBP IgG preabsorbed with synthetic BDP-13 peptide. In each panel, eosinophils are indicated by a filled arrow, and neutrophils are indicated by an open arrow. The unlabeled cells in panel B are mononuclear cells.

![Fig. 5. Immunolocalization of bOBP in peripheral leukocytes.](http://www.jbc.org/)
Figure 6. Northern blot of bovine tissues. Expression of bOBP mRNA in bovine tissues was determined by Northern blot analysis. 20 μg of RNA from bovine bone marrow (BM), small intestine (SI), trachea (Tr), liver (L), and spleen (Sp) was resolved by electrophoresis in a standard 1% agarose, 6% formaldehyde gel. The membrane was sequentially hybridized under stringent conditions with the 32P-labeled full-length bOBP cDNA and mouse GAPDH cDNA.

Figure 7. Antimicrobial activities of bOBP and BDP-13. Agar radial diffusion assays were performed using varied protein concentrations. Activity is expressed as the diameter of clearing of duplicate samples resulting from the application of 5 μl of BDP-13 (○) or bOBP (○) against each organism at the indicated concentrations.

Figure 8. Microbicidal activities of bOBP. Log phase organisms were incubated with varied concentrations of bOBP in pH 7.4 buffer for 2 h at 37 °C before plating. The incubation buffers were as follows: 10 mM PIPES with 5 mM dextrose for L. monocytogenes 9677 and L. monocytogenes 10403s; 10 mM PIPES for C. neoformans 271a; 10 mM Tris-HCl with 5 mM dextrose for S. typhimurium GALe and S. typhimurium SH 6497; and 10 mM Tris for S. aureus 502a. Microbicidal activity was determined by colony counting of plates after 24–48 h incubation.

We selected the term bOBP for this member of the PGRP family because of the fact that it kills some microorganisms that lack peptidoglycan (C. neoformans) or in which the peptidoglycan is obscured by LPS (e.g. S. typhimurium). Moreover, recombinant mouse PGRP reportedly binds LPS (26). Preliminary studies in our laboratory have shown that bOBP binds to each of the microorganisms that it kills, and it also binds to preparations of microbial antigens including lipopolysaccharide and lipoteichoic acid in addition to peptidoglycan. Also, PGRP is reported to interact with the glycan moiety of peptidoglycan (26, 27). Thus, we hypothesize that the binding of bOBP to microbial targets is mediated by oligosaccharide components present in each of these cell surface structures, and that the relative binding affinities are dependent on the composition and stereochemistry of the specific sugar residues.

Thus far, we have detected bOBP in eosinophils and neutrophils but not monocytes, lymphocytes (Fig. 5), or platelets (data not shown), and bOBP mRNA was detected only in bone marrow. Immunocytochemical analysis was suggestive of a granular localization of bOBP in granulocytes (Fig. 5), and this subcellular localization was further supported by the purification of bOBP from cytoplasmic granule preparations of bovine leukocytes. Despite the stronger immunostaining of bOBP in eosinophils (Fig. 5), quantitative HPLC and immunoblotting assays demonstrated that neutrophils and eosinophils contain equal quantity of bOBP per cell (data not shown). Thus, the difference in immunostaining probably reflects differences in antigen presentation in the cytoplasm of the two granulocyte populations.

To our knowledge, bOBP is the only microbicidal granule protein other than bactericidal/permeability-increasing protein and eosinophil cationic protein that is expressed in eosinophils and neutrophils (48). Largely considered effectors of antiparasitic immunity, eosinophils are not recruited to sites of bacterial infection and they possess little bactericidal activity in vitro. This finding raises the possibility that bOBP may contribute to the antiparasitic activities of the eosinophil.

The data presented here provide further evidence for the role of PGRPs in mammalian innate immunity. Specifically, our studies demonstrated that (i) bOBP is bactericidal and fungicidal in vitro at low micromolar concentrations, (ii) bOBP is expressed in the granules of circulating neutrophils and eosinophils, and (iii) it is an ortholog of proteins implicated in the immune functions in insects (25, 27, 28). Although it is apparent that bOBP and other PGRPs recognize microbes by pattern recognition, additional studies are needed to identify the mo-

C. Chace Tydell, Y. D. Tran, J. Yuan, and M. E. Selsted, manuscript in preparation.
lecular determinants of PGRP microbial recognition as well as the mode of PGRP-mediated antimicrobial action.

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