Functional and biochemical characterization of epithelial bactericidal/permeability-increasing protein

Geraldine Canny, Elke Cario, Andreas Lennartsson, Urban Gullberg, Ciara Brennan, Ofier Levy, and Sean P. Colgan.

Functional and biochemical characterization of epithelial bactericidal/permeability-increasing protein. Am J Physiol Gastrointest Liver Physiol 290: G557–G567, 2006. First published November 10, 2005; doi:10.1152/ajpgi.00347.2005.—Epithelial cells of many mucosal organs have adapted to coexist with microbes and microbial products. In general, most studies suggest that epithelial cells benefit from interactions with commensal microorganisms present at the luminal surface. However, potentially injurious molecules found in this microenvironment also have the capacity to elicit local inflammatory responses and even systemic disease. We have recently demonstrated that epithelia cells express the anti-infective molecule bactericidal/permeability-increasing protein (BPI). Here, we extend these findings to examine molecular mechanisms of intestinal epithelial cell (IEC) BPI expression and function. Initial experiments revealed a variance of BPI mRNA and protein expression among various IEC lines. Studies of BPI promoter expression in IECs identified regulatory regions of the BPI promoter and revealed a prominent role for CCAAT/enhancer binding protein and especially Sp1/Sp3 in the basal regulation of BPI. To assess the functional significance of this protein, we generated an IEC line stably transfected with full-length BPI. We demonstrated that, whereas epithelia express markedly less BPI protein than neutrophils, epithelial BPI contributes significantly to bacterial killing and attenuating bacterial-elicited proinflammatory signals. Additional studies in murine tissue ex vivo revealed that BPI is diffusely expressed along the crypt-villous axis and that epithelial BPI levels decrease along the length of the intestine. Taken together, these data confirm the transcriptional regulation of BPI in intestinal epithelia and provide insight into the relevance of BPI as an anti-infective molecule at intestinal surfaces.  

mucosa; infection; inflammation; transcription; intestine

AMONG THE INNATE ANTI-INFECTIVE DEFENSE MOLECULES OF HUMANS is bactericidal/permeability-increasing protein (BPI), a 55- to 60-kDa protein originally found in neutrophil azurophilic granules (62), on the neutrophil cell surface (60), and, to a lesser extent, in specific granules of eosinophils (3). More recently, BPI has been shown to be expressed in epithelia (4) and by fibroblasts (49). BPT’s high affinity for the lipid A region of lipopolysaccharide (LPS) (16) in the gram-negative bacterial outer membrane is followed by a time-dependent penetration of the molecule to the bacterial inner membrane, where damage results in a loss of membrane integrity, dissipation of electrochemical gradients, and bacterial death (39). In addition, BPI is capable of inhibiting all of the many proinflammatory activities of LPS, including the induction of cytokine release, activation of neutrophil oxidase enzymes, and nitric oxide formation (6, 32, 40, 63). This protein can also serve as an opsonin for phagocytosis of gram-negative bacteria by neutrophils (9, 34). BPI’s actions are amplified by extracellular factors including the complement system and secretory phospholipase A2 (10), and members of the desfensi- din antimicrobial peptide families synergistically enhance BPI’s antibacterial activity (33).

A growing number of proteins with primary structural homology to BPI and roles in lipid recognition and host defense are being identified (67). Plasma LPS binding protein (LBQ) is a product of the liver with an anionic net charge that serves to greatly amplify responses to LPS by delivering LPS monomers to a receptor complex containing CD14, myeloid differentiation protein 2 (MD-2), and Toll-like receptor 4 (TLR4) (14, 55). Whereas LBP recruits LPS monomers for delivery to CD14, BPI stabilizes LPS aggregates, thereby preventing LBP-mediated LPS monomer delivery (53). Thus BPI and LBP are functionally antagonistic. It is the COOH-terminal domains of LBQ and BPI that determine the route and host responses to LPS complexes (20). Whereas LBP predominates in resting plasma, the higher affinity of BPI for LPS (15) coupled with the greater concentration of BPI at neutrophil-rich inflammatory sites favors BPI-mediated endotoxin neutralization (43). Homologs of BPI/LBP have recently been described in the head kidney and liver of a teleost (rainbow trout), suggesting that the BPI/LBP family is evolutionarily conserved (19).

The selective and potent action of BPI against gram-negative bacteria and their LPS are fully manifest in biological fluids, including plasma, serum, and whole blood (31, 62). In multiple animal models of gram-negative sepsis and/or endotoxemia, administration of BPI congeners is associated with improved outcome (12, 36). These properties have rendered BPI an attractive target of biopharmaceutical development (31). BPI has proven safe and nonimmunogenic in phase I trials and has demonstrated potent antiendotoxic activity in phase II trials (17). In a large, multinational phase III double-blind, placebo-controlled trial, adjunctive treatment with the recombinant protein consisting of the NH2-terminus of BPI (rBPI21) was associated with reduced complications, including limb ampu-
tion, due to meningococcal sepsis (30). Additional indications for the use of BPI congeners are being actively explored.

Recent studies have identified expression of BPI in human epithelia (4, 44). Here, we define molecular and functional aspects of epithelial BPI. This study is also the first to define the presence of a BPI ortholog in murine epithelia.

MATERIALS AND METHODS

Cell culture. The intestinal epithelial cell lines T84, Caco-2, SW480, and MODE-K were cultured as previously described (8, 51, 56). The clone of HEK-293 cells stably transfected with the human hemagglutinin (HA)-tagged TLR4 gene (passages 15–19; lot no. 2601-293hTLR4HA) was obtained from InvivoGen (San Diego, CA) and maintained as previously described (38). For IL-8 release, a rough strain (obtained from the Salmonella Genetic Stock Center; Calgary, Alberta, Canada) and a smooth strain (14028, from American Type Culture Collection; Rockville, MD) of Salmonella typhimurium were cultured as previously described (4). Bacteria (107) were incubated with wild-type (WT) and BPI-overexpressing cells for 2 h, followed by a washing and then incubation with gentamicin (0.5 mg/ml) to kill extracellular bacteria. Cells were washed again and maintained in medium for the indicated times, after which supernatants were collected, centrifuged to pellet any cellular debris present, and subsequently assayed for IL-8. For experiments using anti-TLR4 antibody (eBioscience; San Diego, CA), 20 µg/ml of the antibody or an isotype-matched control were preincubated with Caco-2 cells before the addition of bacteria and bacterial supernatants. Thereafter, the experiment was performed as previously described. For studies assessing the colocalization of BPI and TLR4, highly purified (99.9% free of DNA and protein) LPS from Escherichia coli serotype R515 (lot no. L12163) and S. typhimurium (lot no. L13725/d) were purchased from Alexis Biochemicals (Grünenberg, Germany).

Transcriptional analysis. Cells were lysed, and RNA was extracted using a RNeasy Protect minikit (Qiagen; Hilden, Germany) according to the manufacturer’s instructions. Reverse transcription was performed using the Iscript kit from Bio-Rad. RT-PCR analysis was performed using the following primer sets. The human BPI forward primer was 5′-GCAACCTGTCTCTGATGGG-3′ and the reverse primer was 5′-AGCCAAATGGGAATATTCTTG-3′, which yielded a 256-bp product. The human β-actin forward primer was 5′-TGAC-CCAGATCATGTTTGAGA-3′ and the reverse primer was 5′-ATGCCATCACGTGTCAG-3′, which yielded a 131-bp product. The PCR contained, in addition to DNA, 1 µM each of the forward and reverse primer, 10 µl of 5× PCR buffer, 1 mM MgSO4, 0.2 mM dNTP, and 5 units Tji enzyme mix in a total volume of 50 µl. Each primer set was amplified using 25–30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s and a final extension of 72°C for 5 min. PCR products were then visualized on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide.

For real-time analysis, the following primers were used. The human BPI forward primer was 5′-CCCTGGAACCATCCTTTC-GAC-3′ and the reverse primer was 5′-CACCACCTAGCATT-TCCA-3′. The human actin forward primer was 5′-CGGCTCCAGGTGAATG-3′ and the reverse primer was 5′-ACTGGAACCGTGTAAGTGACAG-3′. The murine BPI forward primer was 5′-AGATACACCTGGGAAAAGG-3′ and the reverse primer was 5′-GCATCTGCTGTGGTGAATG-3′. The murine actin forward primer was 5′-AACCTTAAGGGCAACGGTAAA-3′ and the reverse primer was 5′-GCAGCAGGATTCTTCTCTCA-3′.

Comparison of gene expression in a semiquantitative manner was performed based on the mathematical model of Pfaf (45).

Transfection and luciferase reporter assays. Reporter assays were performed after overnight transfection of Caco-2 cells with WT and mutated promoter constructs using Polyfect reagent (Qiagen). Luciferase activity was assessed on a TD20/20 luminometer (Turner Designs; Fresno, CA) using the dual-luciferase assay system (Promega; Madison, WI). All activity was normalized with respect to a cotransfected Renilla reporter. In subsets of experiments, transfected Caco-2 cells were exposed to a panel of inflammatory mediators, including IL-1β, IL-4, and TNF-α (all at 20 ng/ml), interferon (IFN)-γ (300 U/ml); phorbol myristate acetate (PMA; 50 nM), ultrapure LPS from Salmonella minnesota (List Laboratories; Campbell, CA; 100 ng/ml), PGE2 (50 nM), and the lipoxin A4 analog 15-epi-16-(para-fluoro)phenoxy-lipoxin A4 (ATLa; 100 nM).

Chromatin immunoprecipitation assay. Confluent Caco-2 cells were cross-linked with 1% formaldehyde for 20 min, harvested, and lysed, and the resulting chromatin was sheared using a F550 microtip cell sonicator (Fisher Scientific). After centrifugation, chromatin-containing supernatants were precleared with a salmon sperm DNA-protein A slurry. The samples were again centrifuged and divided, and each 10 µg of -CCAAT/enhancer binding protein (CEBPβ), anti-Spi antibodies, or control isotype-matched antibodies (Santa Cruz Biotechnology) were added to the supernatant and incubated overnight at 4°C. A further incubation in the presence of protein A-Sepharose beads (Amersham Biosciences; Uppsala, Sweden) was performed. The immunocomplexes were thoroughly washed, and DNA was eluted from the beads, after which the cross-linking was reversed by incubation at 65°C for 6 h. The immunoprecipitated DNA was recovered using a purification kit (Qiagen). PCR was then performed using forward primer 5′-CACAACATATAACACGCG-3′ and reverse primer 5′-AAAGTCTGGGAGGAAATG-3′ for CEβP and forward primer 5′-CACAACATATAACACGCG-3′ and reverse primer 5′-AAAGATCGCTGGGAGAATG-3′ for Sp1/3.

PCR carriers were out for 32 cycles.

Immunocytochemistry and confocal immunofluorescence microscopy. 293hTLR4HA cells were grown overnight in full media (10% FCS) on collagen I-precoated eight-well glass slides (BD Labware; Bedford, MA). Cells were washed once with PBS (without Ca2+/Mg2+) at 37°C, fixed with methanol-acetone (50:50) for 5 min at −20°C, air dried, and washed three times with Tris-buffered saline-Tween. Cells were blocked with normal goat serum (1:100 in PBS) for 60 min at room temperature and incubated with primary antibodies, either a mouse monoclonal to the HA tag (Cell Signaling Technology; Beverly, MA) or a rabbit polyclonal to human BPI (Xoma) at 1:50–1:200 dilutions overnight at 4°C. Alexa Fluor 488-conjugated goat anti-mouse and CY5-conjugated goat anti-rabbit IgG antibodies were used as secondary antibodies (1:100 for 60 min at room temperature). Samples were mounted (Vectashield Mounting Media, Vector Laboratories; Burlingame, CA) and assessed within the next 24 h using a laser-scanning confocal microscope [Plan-Neofluar 63×/1.4 (oil) differential interference contrast objective, zoom 2.0, Zeiss Axiosvert 100M-LSM 510; Jena, Germany]. The multitrack option of the microscope and sequential imaging for each channel were used to eliminate any cross-talk of the chromophores and to ensure reliable quantitation of colocalization. Single-plane optical slices were then processed for double-labeled cells using identical laser and standardized microscope settings (software LSM510 version 3.2, 2048 × 2048 pixels, two channels, 12 bit) and exported to Adobe Photoshop 5.0LE (TIFF). Representative results are shown for the experiment.

Quantitative colocalization analysis. The two channels were merged in the Zeiss LSM510 version 3.2 software during acquisition, allowing qualitative judgments about colocalizing image features labeled with dual-fluorescent dyes (Alexa Fluor 488 and CY5). Two-step image analysis was then performed. First, after a scattergram of raw data of representative double-labeled images in the Zeiss software was created, a morphological filter was applied to remove the noise spikes while maintaining the original contrast ratio between the specific staining and background. A subsequent filter then automatically selected the most contrasting areas, leading to a binary, threshold mask where the colocalized image pixels (yellow) were extracted from the rest of the images. Second, quantitation of the extent of colocalization in the sections was assessed in the Zeiss software by determining the
colocalization coefficient after automatic background correction of each image. A representative result with its specific colocalization coefficient value is shown.

**Generation of cells overexpressing BPI.** A cell line stably overexpressing BPI was generated by transfecting full-length BPI cDNA in a pCDNA3 vector. Clones were selected and propagated in the presence of 800 μg/ml G418 (Invitrogen; Carlsbad, CA). Cells were then routinely cultured in 100 μg/ml G418, and overexpression of BPI was confirmed by PCR and ELISA.

**Bacterial killing assay.** WT and BPI-overexpressing Caco-2 cells (Caco-2-WT and Caco-2-BPI cells, respectively) were grown to confluence on plastic tissue culture-treated plates. Cells were washed once with Hank’s balanced salt solution, and washed S. typhimurium strain 14028 was added to epithelial monolayers at a ratio of 10–20 bacteria per adherent epithelial cell and allowed to incubate for 45 min at 37°C. Parallel samples omitting epithelial cells were used as controls. After the incubation, supernatants were collected, and cells were hypotonically lysed with ice-cold water. The resulting lysates were plated on lburia agar, and colony-forming units were counted the following day. Results were expressed as percent killing compared with controls, which consisted of bacteria alone. No bacterial death was observed in these controls.

**ELISA.** Before the BPI ELISA was performed, cell lysates and epithelial preparations were normalized for protein content using the Bradford assay (Pierce; Rockford, IL). The ELISA plate was coated with goat anti-BPI antibody, covered with an adhesive strip, and left at 4°C overnight. After being washed, the plate was blocked for 60 min at room temperature. Immediately before use, standards ranging from 0.012 to 100 ng/ml were prepared. The plate was washed, and samples and standards were then added. The plate was again covered and incubated for 60 min at 37°C. After this period had elapsed, the wash step was carried out again, the detection antibody was added, and the plate was incubated for 60 min at 37°C. The wash step was repeated, streptavidin-horseradish peroxidase solution was added, and the plate was then covered with tin foil and incubated for 15 min, after which the plate was washed and the substrate solution was added. After color development, the stop solution was added, and the optical density was immediately determined using a microplate reader set to 405 nm. In subsets of experiments, a BPI ELISA (HyCult Biotechnology; The Netherlands) was performed according to the manufacturer’s specifications. Both Xoma and HyCult ELISAs are directed against human BPI but cross-react with the murine ortholog. The IL-8 ELISA was performed as previously described (13).

**Antisera generation, affinity purification, and characterization of the anti-BPI antibody.** Antisera were raised in rabbits injected with 2 mg rBPI21 (courtesy of Dr. Pat Scannon, Xoma). Antisera obtained at 8 and 10 wk, in addition to the terminal bleed, displayed consistently high titers when assayed in a BPI ELISA. Serum was spun at 3,000 g at 4°C to pellet debris. rBPI21 was linked to an affinity purification column using the Aminolink plus immobilization kit (Pierce) according to the manufacturer’s instructions. Antisera were poured across this column, and after a wash, the polyclonal antibody was eluted in 100 mM glycine (pH 2.8).

**Tissue immunohistochemistry.** Staining of murine tissue was performed using paraffin-embedded tissue. After deparaffinization, antigen retrieval was performed using high-pH solution (Dako Cytomation; Carpinteria, CA). After being blocked, sections were incubated with antiserum (raised against a peptide of murine BPI and obtained from A. Lennartsson) or affinity-purified rabbit anti-BPI antibody for 60 min at room temperature. As respective controls, prebleed antiserum and adsorbed antibody were used to determine specificity. A fast red substrate solution (Biogenix; San Ramon, CA) was then applied, after which sections were counterstained and mounted.

**Data analysis.** Results from promoter assays, bacterial killing assays, and ELISAs were analyzed using Student’s t-tests. Values are expressed as means ± SE. P values of <0.05 were considered significant.
detection: 0.1 ng/ml). BPI levels in each of the epithelial lines examined showed protein levels at the limit of detection (0.9 – 3 ng/ml). For comparative purposes, a screen of polymorphonuclear lysates revealed expression levels of 90 ± 5 ng/ml BPI, which approximates our findings by mRNA (e.g., ~50-fold differences in cellular expression levels).

**Functional BPI promoter analysis in epithelia.** Essentially nothing is known about the molecular mechanisms that govern BPI expression in enterocytes. Recent work in leukocytes defined and characterized human BPI promoter activity (28). Using these reporter constructs, we addressed BPI promoter activity in Caco-2 cells. Truncations were generated by making deletions in the 5′-direction (28). Using both full-length (1,100 bp) and truncated (222 and 693 bp) promoter constructs (Fig. 2A), we established that Caco-2 cells express the appropriate transcriptional machinery for BPI expression. With the use of these reagents, it was revealed that basal promoter activity decreased with increasing length, suggesting the likelihood that inhibitory elements exist to control expression in the full-length promoter. We observed that the 222-bp promoter construct possesses the highest activity, correlating with that previously reported (28), implying that critical regulatory elements are located within this region. This cloned promoter has binding sites for a number of well-described transcription factors. By mutating binding sites for the transcription factors C/EBP and Sp1/3 in the 222-bp construct, we determined that each transcription factor contributed to basal transcription in Caco-2 cells. Indeed, as seen in Fig. 2B, mutations encoding changes to binding sites for Sp1/3 and C/EBP resulted in significantly decreased basal promoter activity, with a 82 ± 3%, and 45 ± 3% decrease in activity, respectively (for each, P < 0.01 compared with nonmutated 222-bp promoter).

We extended these studies to address transcription factor binding to the BPI promoter in epithelial cells. For these purposes, we used chromatin immunoprecipitation (ChIP) assays. As shown in Fig. 2, C and D, ChIP analysis demonstrated that C/EBPβ and Sp1/3, respectively, bind to the BPI promoter in Caco-2 cells. Indeed, a PCR product of the expected size for both Sp1/3 (200 bp) and C/EBP (200 bp) was obtained from chromatin samples incubated with the appropriate antibodies. No bands were evident in control IgG immunoprecipitates, and input samples (preimmunoprecipitation) revealed the predicted bands under both PCR conditions. Such results confirmed our mutagenesis results (Fig. 2B) and indicated that Sp1/3 and C/EBP bind directly to the BPI promoter in epithelial cells.

**Expression of BPI in intestinal epithelial cells.** We next examined whether BPI promoter activity was regulated by mediators found in the inflammatory microenvironment. Here, we profiled activity after exposure of Caco-2 cells to a panel of cytokines, lipid mediators, and bacterial products. As shown in

---

**Fig. 2.** Transcriptional activity of BPI promoter constructs in Caco-2 cells. **A:** BPI promoter construct map showing the full-length promoter (1,100 bp) and the location of two truncated constructs (693 and 222 bp). Also depicted are the location of binding sites for the transcription factors C/EBP and Sp1/3. **B:** relative luciferase (Luc) activity of indicated constructs after transient transfection in Caco-2 cells compared with empty PGL3 and a SV40-driven PGL3 promoter (solid bar). Also shown are the influences of the CCAAT/enhancer binding protein (C/EBP) mutation (ΔC/EBP) and Sp1/3 mutation (ΔSp1/3) within the 222-bp construct. Luc activities were calculated relative to those of the co-transfected control plasmid (Renilla) and are expressed as normalized Luc activities ± SE. Results are derived from 3 experiments in each condition. Chromatin immunoprecipitation was used to demonstrate that the transcription factors C/EBPβ (C) and Sp1/3 (D) bind to the BPI promoter in Caco-2 cells. Reaction controls included immunoprecipitations performed by using nonspecific isotype- and species-matched antibodies (IgG CH) and PCR performed using Caco-2 genomic DNA (input). A representative example of 3 independent experiments is shown.
activity (significantly decreased compared with no treatment, H9253/H9251, TNF-
each condition. As indicated, IFN-
normalized Luc activities /H11006 inflammatory mediators. Three cytokines (TNF-
222-bp BPI promoter was screened using a broad range of
Fig. 3, promoter activity in Caco-2 cells transfected with the
After transient transfection of the BPI 222-bp construct, Caco-2 cells were
exposed to media alone (hatched bar) or indicated inflammatory mediators for
Fig. 3. Screen of BPI promoter activity in response to immune mediators. To analyze the subcellular distribution of TLR4 and BPI in
epithelial cells exposed to purified TLR4 ligands, either LPS from E. coli or S. typhimurium, each at a concentration of 1
µg/ml, was added separately to TLR4-HEK-293 cells for
different time periods (15 and 60 min) at 37°C in serum-
Supplement media (10% FCS). As shown by confocal laser microscopy studies, TLR4 and BPI significantly colocalized in
joint cytoplasmic compartments of epithelial cells after 15 min of LPS stimulation. LPS from S. typhimurium (Fig. 4B) in-
duced redistribution of BPI and TLR4 confined to large aggre-
gates of lysosome-like structures, which were redistributed into
multivesicular structures 45 min later. In contrast, E. coli
LPS-induced trafficking of BPI and TLR4 (Fig. 4C) was more scattered, appearing in much smaller but numerous vesicles
broadly dispersed in the cellular cytoplasm. Calculation of the corresponding colocalization coefficient values confirmed that
a major portion of redistributed TLR4 significantly overlapped with BPI in response to both LPS serotypes.

Functional studies using Caco-2-BPI cells. We (4) have previously demonstrated that epithelial BPI contributes to both
bacterial killing and resistance of epithelia to LPS stimulation. In light of the BPI promoter findings above, we further ad-
dressed BPI function in a Caco-2 cell line stably overexpressing
BPI (Caco-2-BPI). This cell line expressed nearly 20-fold
increased BPI mRNA (Fig. 5A) and protein (Fig. 5B: P < 0.01)
compared with WT cells. Taking the protein content of Caco-
2-BPI lysates into account, this could also be expressed as
~6–7 ng BPI/mg total protein, which is in keeping with BPI
levels in the murine small intestine (as depicted in Fig. 6G). It was not possible for us to obtain human intestinal tissue to
assess BPI levels.

We next addressed BPI functional activity. Here, we com-
pared Caco-2-WT cells with Caco-2-BPI cells for their ability
to kill S. typhimurium (strain 14028). As shown in Fig. 5C,
Caco-2-BPI cells displayed an enhanced ability to mediate
bacterial killing compared with Caco-2-WT cells (45 ± 3.5% vs.
20 ± 3% killing at the 45-min time point, P < 0.025).
Further studies revealed that overexpression of BPI significantly attenuates bacterially induced signaling, as shown in Fig. 5D. Indeed, bacterially elicited IL-8 release from Caco-2-BPI cells was significantly diminished compared with Caco-2-WT cells. This inhibition was the case for IL-8 release induced by two different smooth strains of *S. typhimurium* (strains 14028 and 210, 57 ± 8% and 74 ± 5% decrease, respectively, *P* < 0.025 for both). Similar results were observed using a rough strain of *S. typhimurium* (56 ± 5% inhibition, *P* < 0.025). Such inhibition did not require live bacteria, because heat-killed bacteria elicited a robust IL-8 release. This IL-8 release elicited by heat-killed bacteria (see Fig. 5D) was likewise attenuated in Caco-2-BPI compared with Caco-2-WT cells (strains 14028 and 210, 37 ± 6% and 64 ± 5% decrease, respectively, *P* < 0.025 for both). Similar results were observed with gentamicin-killed bacteria (data not shown). As shown in Fig. 6D, both cell lines produced comparable levels of IL-8 in response to IL-1β, an established stimulus for chemokine release from Caco-2 cells (8, 24). Furthermore, preincubation of the cells with functional anti-TLR4 antibody or an isotype-matched control antibody had no effect on the IL-8 release mediated by the 210 strain of *S. typhimurium* or bacterial supernatant (data not shown). This may imply that the chemokine secretion is uncoupled from TLR4 signaling, that receptor levels on Caco-2 cells are too low for the neutralizing antibody to elicit an effect, or that another receptor or signaling pathway is involved.

Expression patterns of BPI in murine intestinal tissue. To examine murine tissue BPI expression, we generated an anti-BPI antibody using rBPI21 (see MATERIALS AND METHODS). Using this antibody, we examined BPI expression in the mouse intestine. As shown in Fig. 6, BPI is prominently expressed in intestinal. As shown in Fig. 6, BPI is prominently expressed in epithelial cells. Overexpression of BPI in Caco-2 cells (Caco-2-BPI cells) was confirmed at mRNA (A) and protein (B) levels by PCR and ELISA, respectively [*significantly increased compared with wild-type Caco-2 cells (Caco-2-WT), *P* < 0.001]. Two functional readouts were used; namely, killing of a smooth strain of *S. typhimurium* (C) and IL-8 release elicited by *S. typhimurium* (D). In each case, Caco-2 cells were grown on plastic and incubated with bacteria, after which cellular lysates were plated and killing was assessed or supernatants were taken at 24 h and assayed for IL-8 by ELISA. In C, overexpression of BPI enhanced bacterial killing compared with Caco-2-WT cells (*significantly increased compared with Caco-2-WT cells, *P* < 0.025). D: analysis of IL-8 release from Caco-2-WT cells (solid bars) and Caco-2-BPI cells (open bars) after stimulation with live or heat-killed (HK) *S. typhimurium* (strains 210 and 14028) or IL-1β (10 ng/ml). Overexpression of BPI resulted in attenuated IL-8 release by both live and HK bacteria (*P* < 0.025 compared with Caco-2-WT cells).

Fig. 4. LPS induces significant endosomal colocalization of BPI with Toll-like receptor 4 (TLR4) in epithelial cells. HEK-293-TLR4-hemagglutinin (HA) cells grown on collagen I-coated glass slides were stimulated without (A) or with LPS (1 μg/ml) from *Salmonella typhimurium* (B) or *Escherichia coli* (C) for 15 or 60 min, fixed with acetone-methanol, stained with anti-BPI and anti-HA antibodies, and then analyzed by immunofluorescence confocal laser microscopy. BPI, anti-BPI (red) antibody detected by CY5-conjugated secondary antibody; HA-TLR4, anti-HA (green) antibody detected by Alexa Fluor 488-conjugated secondary antibody; merged, merged BPI and HA-TLR4 antibodies; extracted, extracted colocalization areas. (Image acquisition details are magnification ×63/1.4 oil; zoom 2.0; 2048 × 2048 pixels; 2 channels; 12 bit.) A: BPI only marginally colocalizes with TLR4 under steady-state conditions (colocalization coefficient value: 0.240). B: *S. typhimurium* LPS induces significant colocalization of BPI and TLR4 confined to large lysosome-like aggregates (colocalization coefficient values: 15 min, 0.513; and 60 min, 0.480). C: *E. coli* LPS induces dispersed redistribution of BPI and TLR4 colocalizing in small vesicular structures (colocalization coefficient values: 15 min, 0.392; and 60 min, 0.570).
mouse intestinal epithelial cells. With the use of both our own polyclonal anti-BPI antibody and previously reported BPI antisera (29), localization of BPI was evident in both villous and crypt epithelial cells, and, similar to human tissue, BPI was diffusely expressed throughout the cytoplasm of individual epithelial cells.

As part of this analysis, we also examined epithelial BPI protein levels. To do this, mouse epithelial cells were isolated from various anatomic regions along the length of the mouse intestinal tract, and lysates were analyzed for BPI content by ELISA. As shown in Fig. 6, BPI ELISA analysis revealed that murine epithelial BPI levels decrease at distal regions of the intestine. Indeed, BPI levels decreased by nearly 65% from the proximal small intestine to the colon.

**DISCUSSION**

We (4) have previously demonstrated the expression of functional BPI in mucosal epithelial cells. This cationic protein, with its potent ability to neutralize LPS and mediate killing of a wide range of gram-negative bacteria, plays a potentially significant role at the epithelial/bacterial interface. In the present study, we examined the regulation of BPI in intestinal epithelia and further elucidated its functional significance.

From our promoter assay experiments, Caco-2 cells possess the appropriate transcriptional machinery to express BPI. Further analysis suggested that Sp1/3 and C/EBP likely contribute to this regulation, particularly Sp1/3. Mutation of the Sp1/3 site nearly ablated expression of the BPI promoter. At present, we do not know whether this regulation occurs predominantly through Sp1, Sp3, or both. Whereas Sp1 and Sp3 have similar structures and high homology in their DNA-binding domains, these two transcription factors can have striking differences in function (35).

BPI promoter regulation in intestinal epithelial cells was surprisingly unresponsive to external stimuli. By profiling a broad range of inflammatory mediators, we found minimal changes from baseline expression. Similarly, using SW480 cells, which have previously been used to characterize LPS signaling (51), we observed that BPI expression is not subject to regulation by LPS itself, negating the existence of a regulatory loop. Such findings may indicate that this protein is involved in homeostasis, and it would therefore appear that antimicrobial peptides and proteins may be expressed in specific intestinal locations under certain conditions. Evidently, this is also dependent on the inherent responsiveness and receptor expression on enterocytes. For example, it has recently been demonstrated that β-defensin 2 expression is up-regulated in SW480 cells by LPS in a TLR4-dependent manner (57). However, although LPS does not appear to regulate the expression of BPI, the localization of this protein is altered upon exposure of epithelia to LPS. As recently demonstrated,
TLR4 is distinctly present at the cell surface of polarized intestinal epithelial cell monolayers (5). When stimulated with LPS from *E. coli*, TLR4 redistributes to vesicular structures in the cytoplasm. Electron microscopy has shown that some of these structures resemble multivesicular lysosomes, whereas smooth, membrane-bound vesicles resemble those of endosomes (5). We now confirm this previous finding of LPS-induced TLR4 redistribution in another epithelial cell line and further demonstrate, for the first time, that TLR4 significantly colocalizes with BPI in such endosomal-lysosomal structures in response to LPS stimulation. This influence of LPS was evident as early as 15 min of exposure and was more impressive after stimulation with LPS from *S. typhimurium* than with LPS from *E. coli*. It has recently been shown that LPS is actively taken up by intestinal epithelial cells and that endocytosed LPS significantly colocalizes with lysosomal structures (5, 51). Future studies will be necessary to determine whether these TLR4/BPI-positive vesicles may also carry endocytosed LPS as cargo, to clarify the signaling mechanism of endotoxin detoxification through BPI via TLR4, and what the functional consequences of this phenomenon are.

The expression of proteins involved in LPS recognition in the intestine is an area of intense investigation. In this regard, it is interesting to note that LBP, a member of the same protein family as BPI, is apically secreted by Caco-2 cells in response to cytokines (59). Naturally, differences in cell line clones, culture methods, and assay sensitivity must be taken into account when these results are interpreted. Vreugdenhil et al. (59) also demonstrated that the presence of LBP in intestinal mucus was significantly enhanced in mice after endotoxin challenge. The functional influences of BPI and LBP have been traditionally considered as opposing, with LBP enhancing endotoxin signaling and BPI inhibiting such responses (11). Indeed, BPI has a much higher affinity for LPS than does LBP (15). However, an older study (54) and a more recent report (58) have shown a potential anti-inflammatory role for LBP, which, at high concentrations, serves to detoxify LPS by enhancing its delivery to lipoproteins and chylomicrons. The relevance of this phenomena to intestinal immunity remains unclear. LPS, a mediator long perceived as proinflammatory, is therefore a subject of continuing investigation.

An elegant recent study (48) demonstrated that the recognition of commensal bacteria by TLRs is necessary for protection against gut injury and associated mortality. Moreover, this protective effect could be mediated by LPS alone, provoking the questions of what the method of recognition and exact function of endotoxin in the intestinal milieu are. Clearly, such processes are finely regulated, and LPS recognition and formation of the TLR complex are not fully understood.

As part of these studies, we generated a Caco-2 intestinal epithelial cell line that overexpresses BPI. Here, we examined the contribution of BPI toward killing a smooth strain of *S. typhimurium* as well as the influence of BPI on bacterial-elicited chemokine (IL-8) release from intestinal epithelia. Several reports have shown that IL-8 expression is upregulated in inflammatory bowel disease (IBD), and tissue expression was demonstrated to correlate with the degree of inflammation (2, 7, 21–23, 25, 41, 42). Notably, this chemokine accounted for most of the leukocyte chemotactic activity that could be extracted from the inflamed colon (1, 18, 26, 47). BPI overexpression translates into a protective epithelial response, because studies comparing the bactericidal capacity of Caco-2-WT and Caco-2-BPI cells revealed significant increases in killing of a smooth strain of *S. typhimurium*. Moreover, *Salmonella*-induced release of IL-8 by Caco-2-BPI cells was significantly diminished compared with Caco-2-WT cells, indicating that BPI enhances bacterial killing and attenuates bacterial-elicited signal transduction in epithelia. In an attempt to determine whether such differences were explained by increased killing of bacteria, we used dead bacteria to elicit IL-8 signaling. These experiments indicated that both heat-killed and gentamicin-killed bacteria elicit responses and that both responses are also inhibited by overexpression of BPI. Thus the attenuation of IL-8 release by BPI appears to be independent of bacterial killing. With the use of IL-1β, a well-established stimulus for IL-8 release from Caco-2 cells (8, 24), Caco-2-BPI cells released similar amounts of this chemokine to Caco-2-WT cells, indicating that this difference is not reflective of a signaling defect in Caco-2-BPI cells.

This is the first report demonstrating expression of a BPI ortholog in murine intestinal epithelium. Although mice were previously thought not to express BPI, the gene is located on chromosome 2, and a recent study (29) has established that a BPI ortholog is indeed present in murine neutrophils and Sertoli cells. Moreover, we observed that BPI is expressed along the murine intestine, being present in the small intestine, cecum, and colon. The staining pattern we observed in murine enterocytes would appear to be predominantly cytoplasmic, although surface expression cannot be ruled out given the limits of histological resolution. It is noteworthy that BPI expression levels decreased with increasing distance along the intestinal tract (i.e., highest in the small intestine and lowest in colon). This pattern of expression inversely correlates with the level of bacterial colonization along this same tract (i.e., lowest in the small intestine and highest in colon). The present study therefore identifies a previously unappreciated facet of innate immunity in murine enterocytes, and it is tempting to speculate as to whether BPI contributes to the maintenance of normal flora in the intestine. Further studies are necessary to determine whether BPI plays a role in dampening bacteria- and/or LPS-induced inflammation in this environment.

The hypothesis that human IBD might somehow be related to normal bacterial flora was first proposed three decades ago (50). In the intervening time, a significant amount of information has been gathered from animal and human studies supporting the concept that a dysregulated response to antigen(s) secondary to a failure of normal immunoregulatory mechanisms (58), and the observations that the ability of the epithelium to “hold back” flora in IBD may be abrogated (52), it is desirable to elucidate the contribution of proteins such as BPI to intestinal immunity. Moreover, recent data are shedding light on the influence of decreased expression of antimicrobial peptides and proteins in the intestine. It has been postulated that Crohn’s disease is a defensin deficiency syndrome (61), and, interestingly, a polymorphism in the BPI gene has also been associated with Crohn’s disease (27). The impact of these facets of innate intestinal immunity in health and disease is therefore a subject of continuing investigation.
ACKNOWLEDGMENTS

The authors sincerely thank Ryan Doster for contributions to this work, Prof. Charles Serhan for contributing ATLa, and Dr. Pat Scannon of Xoma Corporation for rBP21 and anti-BPI antibody.

GRANTS

This work was supported by National Institutes of Health Grants DK-065768 and DE-016191 (to S. P. Colgan), by a grant from the Crohn’s and Colitis Foundation of America (to G. Canny), and by Deutsche Forschungsgemeinschaft Grant Ca226/4-1 (to E. Cario).

REFERENCES


