The region around residue 115 of human bactericidal/permeability-increasing protein is not involved in lipopolysaccharide binding or bactericidal activity

Chemical synthesis and expression of a gene coding for the active domain and characterization of recombinant proteins

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Bactericidal/permeability-increasing protein (BPI) is a potent antimicrobial agent produced by polymorphonuclear leucocytes that specifically interacts with and kills Gram-negative bacteria. An 825 bp gene determining the bactericidal N-terminal domain of human BPI was chemically synthesized and expressed as inclusion bodies in *Escherichia coli*. The recombinant polypeptide, BPI', was solubilized and conditions under which it folded to give the active protein were determined. Folding was critically dependent on the urea and salt concentrations as well as the pH. BPI' bound with high affinity to *Salmonella typhimurium* cells (apparent $K_d = 36$ nM), permeabilized their outer membranes to actinomycin D, specifically activated a synovial fluid phospholipase A_2 and showed potent bactericidal activity. In contrast with the native protein, however, it could not be efficiently released from the cell surface by the addition of high

concentrations of Mg^{2+} ions. Pre-incubation of the protein with lipopolysaccharide or trypsin prevented cytotoxicity. However, boiling BPI' immediately before its addition to cells did not block its bactericidal activity, suggesting that it may be able to function even when presented to cells in an unfolded form. A BPI' derivative, containing a 13-residue foreign antigenic determinant genetically inserted between Ala¹¹⁵ and Asp¹¹⁶, was also produced. The derivative was functional in the above assays and bound with high affinity to *S. typhimurium* (apparent $K_d = 74$ nM). These results imply that the region defined by these residues is not involved in the lipopolysaccharide-binding or bactericidal activities of BPI. The availability of functional, non-glycosylated recombinant derivatives of BPI should greatly aid detailed studies on its structure, interactions with lipopolysaccharide and mechanism of action.

INTRODUCTION

Polymorphonuclear leucocytes play a central role in the defence of a host against invading bacteria, employing both oxidative and non-oxidative killing mechanisms [1–3]. An important component of the latter is bactericidal/permeability-increasing protein (BPI), a potent antimicrobial agent that is highly cytotoxic towards many Gram-negative bacteria but that has no appreciable activity towards Gram-positive bacteria, fungi or mammalian cells [3–5]. BPI is a cationic polypeptide of 57 kDa that is probably identical to the bactericidal protein (BP) and the 57 kDa cationic antimicrobial protein (CAP57) isolated by other groups [6,7].

BPI binds avidly to the lipopolysaccharide (LPS, also known as endotoxin) molecules present on the external face of the outer membranes of susceptible bacteria and causes an irreversible loss of cell viability within 30 s at 37 °C [8–10]. It also permeabilizes the membrane to a range of hydrophobic compounds and selectively activates certain phospholipases A_2 and peptidoglycan-degrading enzymes [3,4,11]. Despite these effects, however, cells maintain their capacity for macromolecular synthesis for at least 1 h after BPI addition and, while the available evidence suggests that the cytoplasmic membrane is the ultimate target for BPI, the precise molecular mechanism of killing of bacteria remains elusive [4,5,7,12].

The cloning and sequence analysis of the cDNA for BPI strongly suggests that the protein is organized into two domains

[13]. The N-terminal half of the polypeptide contains many basic and hydrophilic residues whereas the C-terminal half has several potential transmembrane regions that may allow the protein to associate with the neutrophil granule membrane. Studies with proteolytic fragments of the native protein also suggest that the protein is bipartite and have shown that the major LPS-binding and cytotoxic activities of BPI reside in an N-terminal 25 kDa fragment [14]. In view of this data, it has been proposed that the bactericidal N-terminal fragment may be functional *in vivo*, following its release by proteolytic cleavage of the holoprotein [13].

With the discovery that BPI neutralizes the endotoxic properties of LPS [14,15], there has been considerable interest in using the protein to treat Gram-negative bacteraemia and septic shock. The availability of large quantities of a recombinant version of the protein, amenable to site-directed mutagenesis, would clearly aid such studies and would allow key questions about the structure and mechanism of action of BPI to be addressed. A non-glycosylated form of the protein, such as can be produced by *Escherichia coli*, might also more readily yield crystals, thereby allowing X-ray crystallographic analysis of the active domain of BPI.

In the present paper, we describe the construction of a synthetic gene encoding the bactericidal domain of BPI (termed BPI'), its expression in *E. coli* and solubilization studies allowing the recovery of the functional folded protein. Analysis of the recombinant BPI' protein, and a derivative carrying a foreign

Abbreviations used: BPI, bactericidal/permeability-increasing protein; BPI', recombinant BPI; LPS, lipopolysaccharide; LB, Luria-Bertani medium; IPTG, isopropyl β -D-thiogalactopyranoside.

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antigenic determinant inserted between residues 115 and 116, indicates that both retain the properties characteristic of the native protein and hence should be invaluable for detailed structural and functional studies.

MATERIALS AND METHODS

Bacterial strains

The *E. coli* strains used for cloning and expression were MV1190 [$\Delta(lac-proAB)$, *thi*, *supE*, $\Delta(srl-recA)306::Tn10$ (*tet^r*) (F' *traD36*, *proAB*, *lacI^a* ZAM15)] (from Bio-Rad Laboratories) and BL21 (DE3) [F⁻, *ompT*, *r_B*⁻*m_B*⁻] [16]. *E. coli* C600 [*thi*, *thr*, *leuB6*, *lacY1*, *tonA21*, *supE44*] and *Salmonella typhimurium* SL761 [*ade*, *ile*, *pro*, *fla*, *rpsL*, *rfaC*], with LPS of the R_a and R_c chemotypes respectively, were employed for the bactericidal and outer membrane permeability assays, whereas *E. coli* CE1303 [*bio*, *pldA*, *recA56*, *endA*] was used for the phospholipase assay [17,18]. Cells were cultivated in Luria-Bertani (LB) medium supplemented, where appropriate, with the relevant antibiotic or, for [³⁵S]methionine labelling of BPI', in M9 minimum medium supplemented with 1% (w/v) glucose and 0.01% Methionine Assay Medium (Difco) [19].

Oligonucleotides and reagents

Oligonucleotides were synthesized on an ABI DNA synthesizer using conventional β -cyanoethyl phosphoramidite chemistry and either used without further treatment following deprotection (for oligonucleotides < 200 bases) or purified by electrophoresis on 20% (w/v) polyacrylamide/8 M urea gels [20]. PCR primers 1, 2, 3 and 4 had the following respective sequences: 5'-GACTAAGGTTCGACGACTAAGATCG-3'; 5'-GCGGCCGCATGCTCTAGATGGCC-3'; 5'-GACTCCGCGTCGACAGATC-3'; 5'-CCACAGAATCGATCTTAG-3'. The double-stranded synthetic DNA fragment coding for the C3 epitope of the VP1 coat protein of type-1 polio virus has been described previously [21]. The fragment was excised from plasmid pIT1 and inserted in-frame into the unique *Bgl*II site within the coding region of the BPI' gene to produce plasmid pSQ90. *E. coli* LPS (R_a chemotype), fatty acid-free BSA and pig pancreatic phospholipase A₂ were purchased from Sigma. [1-¹⁴C]Oleic acid and [³⁵S]methionine were from Amersham International whereas TRANS ³⁵S-LABEL (a mixture of [³⁵S]Cys and [³⁵S]Met) was purchased from ICN.

PCRs and gene assembly

To minimize the number of manipulations required for gene assembly, long (137–277 base) oligonucleotides containing specific segments of the gene were synthesized. Although these were present in quantities that precluded conventional purification, they were readily amplified using the PCR [22,23]. The oligonucleotides were designed with flanking universal sequences to allow PCR amplification using standard primer combinations. Each template oligonucleotide was first converted into the double-stranded form using a primer complementary to its 3' end (i.e. primers 2 or 4) and then amplified using the same primer in combination with a primer complementary to the 3' end of the second strand (i.e. primers 1 or 3). Each PCR contained 100 pg–10 ng of the template oligonucleotide, 10–40 nmol each of the appropriate combination of primers, 20 mM Tris/HCl (pH 8.3 at 20 °C), 1.5 mM MgCl₂, 25 mM KCl, 50 μ M of each

dNTP and 2.5 units of *Taq* polymerase. The PCRs were carried out for 25–40 cycles on a Techne PHC-1 thermal cycler using the following conditions: 94 °C, 1 min; 42–55 °C, 2 min; 72 °C, 3 min. The amplified fragments were then cleaved with the appropriate combination of restriction enzymes, to remove the flanking universal sequences, and cloned into M13mp19 or an M13mp19 derivative containing a previously synthesized portion of the BPI gene (see text for further details). The final assembled construct was sequenced completely on both strands to confirm that it corresponded exactly to the designed sequence.

Expression of the BPI gene in *E. coli*

The synthetic BPI' and BPI'/C3 genes were excised from the replicating form of the M13 vector, using *Bam*HI, and recloned into the expression vector pET11 [24], to produce plasmids pSQ87 and pSQ90, in which their expression was driven by the T7 gene 10 promoter. Efficient regulation of the promoter was achieved by use of *E. coli* BL21 (DE3) which has a λ lysogen bearing *lacI* and the gene for T7 RNA polymerase under the control of the *lacUV5* promoter. Glycerol stocks of *E. coli* BL21-(DE3)(pSQ87) or (pSQ90) were diluted 1 in 500 into fresh LB medium (supplemented with ampicillin at 100 μ g/ml, final concentration) and grown with good aeration at 37 °C to an *A*₆₀₀ value of 0.5–0.7. Cultures were induced by the addition of isopropyl β -D-thiogalactopyranoside (IPTG; 0.7 mM, final concentration) and further incubation for 6–16 h.

Purification and refolding of BPI' protein

Induced cells from 2 litres of culture were harvested at 6000 *g* at 4 °C for 15 min. The pellet was washed once with lysis buffer [20 mM Tris/HCl (pH 8.0), 5 mM Na₂EDTA, 0.5 mM phenylmethanesulphonyl fluoride] and resuspended in the same buffer (30 ml). The cells were then broken by two passages through a French press at 1.38 $\times$ 10 kPa followed by brief sonication (20 s on, 20 s off, 5 cycles) using an MSE Soniprep 150. Inclusion bodies were collected at 12000 *g* at 4 °C for 20 min, washed with water and resuspended in lysis buffer (30 ml) containing 0.2% (w/v) lysozyme. The suspension was incubated at room temperature for 30 min with gentle stirring and sodium deoxycholate, pancreatic deoxyribonuclease I and ribonuclease A were then added to give final concentrations of 2% (w/v), 5 μ g/ml and 5 μ g/ml respectively. The suspension was incubated for a further 30 min before the insoluble protein fraction was collected by centrifugation for 20 min at 12000 *g* at room temperature and washed with 50 mM Tris/HCl (pH 8.0)/50 mM NaCl/5 mM EDTA (sodium salt)/10% (v/v) Triton X-100 and 4 M urea/50 mM citrate/phosphate buffer (pH 6.0). The proteins were dissolved in 8 M urea/50 mM citrate/phosphate buffer (pH 6.0)/0.1% 2-mercaptoethanol (Buffer A) and the solution was cleared by centrifugation at 17000 *g* at 4 °C for 20 min.

Subsequent f.p.l.c. used a Mono S HR 5/5 column (Pharmacia) equilibrated in Buffer A. Elution of BPI' was achieved with the same buffer and a 0–1 M NaCl gradient at a flow rate of 0.3 ml/min. The BPI' and BPI'/C3 proteins, which were eluted at 0.4–0.6 M NaCl, were collected and diluted with Buffer A containing 0.5 M NaCl to give a protein concentration of 20–50 μ g/ml. The proteins were then refolded by dialysis against 50 mM citrate/phosphate buffer (pH 4.0) for 36 h with two buffer changes. After refolding, the protein solution was cleared by centrifugation at 17000 *g* for 20 min at 4 °C and concentrated by ultrafiltration with a Diaflo PM10 filter.

Amino acid sequence analysis of BPI' protein

The N-terminal sequence analysis was performed by sequential Edman degradation on an ABI 477 pulsed-liquid sequencer. Phenylthiohydantoin derivatives were analysed with an on-line ABI 120A detector.

Purification of native BPI protein from neutrophils

Human BPI protein was purified from a neutrophil-enriched fraction from bone marrow (obtained by leukophoresis), using the method of Mannion et al. [9].

Assay for bactericidal activity

E. coli C600 or *S. typhimurium* SL761 were grown to mid-exponential phase, washed in 0.9 % NaCl and resuspended in the same solution at a density of about 1×10^8 cells per ml. An aliquot (10 μ l) of the cell suspension was added to 90 μ l of 20 mM phosphate buffer (pH 6.0) containing BPI' or BPI'/C3 and the mixture was incubated for 30 min at 37 °C. Dilutions were then plated on LB agar plates and scored for survivors following overnight incubation at 37 °C. Where necessary the purified recombinant proteins were pre-incubated with trypsin or *E. coli* R_s LPS before the addition of cells. Bactericidal activity was expressed as the percentage of the number of colony-forming units obtained with cells without the BPI' or BPI'/C3 proteins.

SDS/PAGE and immunoblot analysis

SDS/PAGE and immunoblotting of proteins were performed as described previously [21]. Where necessary, Coomassie Blue-stained gels were scanned using a Joyce-Loebl Chromoscan 3 densitometer. The anti-C3 monoclonal antibody used was kindly supplied by Dr. S. van der Werf, Institut Pasteur, Paris, France.

Protein determination

Total protein was measured using the bicinchoninic acid assay as described previously [24].

Fluorescence experiments

Fluorescence measurements were carried out using an Hitachi F-2000 fluorimeter. To monitor denaturation, BPI' or BPI'/C3 (10 μ g/ml) were incubated with various concentrations of urea for 1 h at room temperature. The fluorescence emission spectra were then measured (λ_{ex} , 280 nm). The kinetics of renaturation of the proteins were determined by denaturation in 8 M urea and subsequent 200-fold dilution into 50 mM citrate/phosphate buffer (pH 4.0). The change in fluorescence intensity at 355 nm (λ_{ex} , 280 nm) were then recorded at intervals up to 180 min. The final protein concentration was 2 μ g/ml.

Assay for activation of phospholipid hydrolysis

Activation of phospholipid hydrolysis by a recombinant form of human synovial fluid phospholipase A₂ was measured essentially as described by Elsbach and Weiss [25]. Briefly, *E. coli* CE1303 cells were labelled with [1-¹⁴C]oleic acid (0.2 μ Ci/ml), washed

with 1 % (w/v) BSA and resuspended in phosphate-buffered nutrient broth (pH 7.2). Phospholipase A₂ activity was determined in a 250 μ l reaction mixture containing 2.5×10^7 cells (10000 c.p.m.), 0.3 % (w/v) fatty acid-free BSA in phosphate-buffered nutrient broth. The mixture was allowed to equilibrate at 37 °C before the addition of native or recombinant BPI (1 μ g) and phospholipase A₂ (0–400 ng). Following incubation at 37 °C for a further 60 min, the reaction was stopped by spinning the mixture in a microcentrifuge for 4 min and transferring the supernatant, containing the released ¹⁴C-labelled fatty acids, to a fresh tube. Radioactivity was measured using a liquid-scintillation counter. Pig pancreatic phospholipase A₂, which has previously been shown to be non-responsive to BPI [11], was included as a negative control.

Assay for BPI' and BPI'/C3 binding to cells

The BPI' or BPI'/C3 proteins, labelled *in vivo* with [³⁵S]methionine and purified as described above, were assayed for binding to *S. typhimurium* SL761 cells as described previously, except that the incubation buffer was 20 mM citrate/phosphate, pH 4.0 [12]. Bound and unbound ³⁵S-labelled proteins were determined in triplicate by counting the radioactivity in washed bacteria and supernatants using a Phillips PW4700 scintillation counter. The recovery of added ³⁵S label was invariably > 80 %. The total number of cells present in the binding assay was determined by using a counting chamber and light microscopy.

Measurement of outer membrane permeability

The effect of BPI' or BPI'/C3 on the permeability properties of the *E. coli* outer membrane was measured using a modified version of the previously described actinomycin D assay [4]. Briefly, *E. coli* and *S. typhimurium* cells were grown to mid-exponential phase, washed and resuspended in 0.9 % NaCl at a density of about 1×10^8 cells per ml. 1×10^6 cells were added to BPI' or BPI'/C3 protein (4–10 μ g, in 20 mM phosphate buffer, pH 6.0; final volume 100 μ l) and incubated for 15 min at 37 °C. Actinomycin D (12.5 μ g) was then added to the incubation mixture and, after a 15 min incubation at 37 °C, TRANS ³⁵S-LABEL (10 μ M, 10 μ Ci) was added. Each mixture was incubated at the same temperature for a further 30 min before the reaction was stopped by the addition of 10 vol. of ice-cold 100 % trichloroacetic acid. Cold trichloroacetic acid-precipitable material was collected on Whatman G/FC filters using a vacuum manifold and the radioactivity counted using a Phillips PW4700 scintillation counter.

RESULTS

Construction of a synthetic gene specifying the bactericidal domain of BPI

Using the cDNA sequence for the BPI gene as a guide, an 825 bp synthetic gene determining the major LPS-binding and bactericidal domain (residues 1–249 of BPI; [13,14]) was constructed (Figure 1). The gene used the codons most commonly found in highly expressed *E. coli* proteins and also contained a ribosome-binding site, derived from gene 10 of bacteriophage T7, and an ATG start codon to allow translation of the corresponding mRNA [26,27]. Where possible, unique restriction enzyme sites were incorporated to facilitate gene assembly and subsequent mutagenesis. Oligonucleotides containing a defined segment of the gene were synthesized with flanking sequences complementary to PCR primers. This permitted the production by PCR of full-length double-stranded fragments that were then inserted

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10      20      30      40      50      60
ATCCTCTAGAGTCGACAATTTTGGTTTAACTTTAAGAGGAGATATATCATGGTTAACCCA
      gcc c t
      METValAsnPro

70      80      90      100     110     120
GGTGTGTGTTGTCGTATCTCTCAAAAGGGTCTGGACTACGCTTCTCAACAAGGTACCGCT
c c g ca g c g c c cagc g g g g c
GlyValValValArgIleSerGlnLysGlyLeuAspTyrAlaSerGlnGlnGlyThrAla

130     140     150     160     170     180
GCTCTGCAAAAGGAAGTGAAGCGTATCAAGATCCAGACTACTCTGACTCTTTCAAGATC
g g g a g t t t a agc t
AlaLeuGlnLysGluLeuLysArgIleLysIleProAspTyrSerAspSerPheLysIle

190     200     210     220     230     240
AAGCACCTCGGTAAAGGCTCACTACTTTCTACTCTATGGATATCCGTAATTCACCTG
t t g g t tagc agc c t
LysHisLeuGlyLysGlyHisTyrSerPheTyrSerMetAspIleArgGluPheGlnLeu

250     260     270     280     290     300
CCATCTTCTCAAAATCTCTATGGTTCCAAAGCTTGGTCTGAAGTTCTCTATCTCTAACGCT
cag c g aagc g c t g c t c agc c
ProSerSerGlnIleSerMetValProAsnValGlyLeuLysPheSerIleSerAsnAla

310     320     330     340     350     360
AACATCAAAATCTCTGGTAAGTGAAGGCTCAAAAGCGTTTCTTAAAGTGTCTGGTAAC
t g agc g a a a a a a a agc c t
AsnIleLysIleSerGlyLysTrpLysAlaGlnLysArgPheLeuLysMetSerGlyAsn

370     380     390     400     410     420
TTCGACCTGTCTATCGAAGGTATGCTATCTCCGACAGATCTGAAGCTGGGTCTTAACCCA
t agc a c c t g t cag c
PheAspLeuSerIleGluGlyMetSerIleSerAlaAspLeuLysLeuGlySerAsnPro

430     440     450     460     470     480
ACTTCTGGTAAGCCAATATCACTGCAGCTCTTGTCTCTACATCAACTCTGTTTAC
g a c c c c t c agc cagcagc ag c
ThrSerGlyLysProThrIleThrCysSerSerCysSerHisIleAsnSerValHis

490     500     510     520     530     540
GTGCACATCTCTAAGTCTAAGTTGGTGGCTGATCCAAGTGTCCACAAGAAGATCGAA
a agc a c g c c c a a a t g
ValHisIleSerLysSerLysValGlyTrpLeuIleGlnLeuPheHisLysLysIleGlu

550     560     570     580     590     600
TCTGCTCTGCGTAACAAGATGAACCTCAAGTTTGTGAAAAGGTTACCAACTCTGTTTCT
g t a agc g c c g a g t a c
SerAlaLeuArgAsnLysMetAsnSerGlnValCysGluLysValThrAsnSerValSer

610     620     630     640     650     660
TCTAAGCTTCAACCACTTCCAAACTCTGCCAGTCATGACTAAGATCGATTCTGTGGCT
c g t t t g a a c a a a
SerLysLeuGlnProTyrPheGlnThrLeuProValMetThrLysIleAspSerValAla

670     680     690     700     710     720
GGTATCAACTACGGTCTGGTGTCTCCACCACTACTGCTGAGACTCTGGACGTTCAA
a t g a t a c g c c t a g
GlyIleAsnTyrGlyLeuValAlaProProAlaThrThrAlaGluThrLeuAspValGln

730     740     750     760     770     780
ATGAAGGGTGAATTTCTACTCTGAAAACCAACCAACCAACCACTTCGCTCCACCAAGT
g g t ag g t t c t t c t t
MetLysGlyGluPheTyrSerGluAsnHisHisAsnProProProPheAlaProProVal

790     800     810     820
ATGGAATTCACAGTGCTTAAATGGCCATCTAGAGCATGCGGCCGC
g t c c t gac gcatggt tacctg t
MetGluPheProAlaAla---

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Figure 1 Nucleotide sequence of the BPI' gene and the predicted amino acid sequence of the BPI' polypeptide

Nucleotides that differ in the original cDNA sequence are shown as lower-case letters beneath the sequence of the chemically synthesized gene. The ribosome-binding site from gene 10 of phage T7 is underlined and the site of insertion of the DNA fragment coding for the 13-residue C3 epitope of polio virus is shown by the vertical arrow. As amino acid sequencing showed that the initiating formyl-methionine was not removed, the BPI' protein is predicted to be 250 residues in length and have a calculated molecular mass of 27 548 Da. The DNA sequence has been deposited with the EMBL Data Library (submitted to the EMBL database, accession number X68790 HSBPIP).

Figure 2 Expression and purification of the BPI' and BPI'/C3 proteins

The figure shows an SDS/13% (w/v) polyacrylamide gel stained with Coomassie Brilliant Blue. Tracks 1–3 contain total SDS-soluble protein fractions and tracks 4–6 contain proteins present in the washed inclusion bodies. The final two tracks show the BPI' and BPI'/C3 proteins after f.p.l.c. purification and refolding. Tracks 1, 2, 4, 5 and 7 contain samples from *E. coli* BL21 (DE3) with pSQ87 (encoding BPI') whereas tracks 3, 6 and 8 contain samples from *E. coli* BL21 (DE3) with pSQ89 (coding for BPI'/C3). With the exception of samples shown in tracks 1 and 4 all were from cells induced with IPTG. Track M contains molecular markers (masses are given on the side in kDa).

sequentially into an M13 vector to achieve the step-wise assembly of the gene. Besides constructing a gene coding for BPI', we also took advantage of a unique *Bgl*II site within the open reading frame to construct a derivative specifying a 13-residue foreign antigen determinant, the C3 epitope of the VP1 coat protein of polio virus [28], genetically inserted between Ala¹¹⁵ and Asp¹¹⁶ (using numbering for native BPI). As synthesis of the BPI'/C3 polypeptide could be readily assayed using an anti-C3 monoclonal antibody, conditions for expression of this derivative were optimized before similar experiments with BPI'.

Expression of the BPI' protein in *E. coli* and its isolation and refolding

It was anticipated that the soluble form of the BPI protein might be deleterious to *E. coli*. However, expression of BPI' by pSQ87 was sufficiently well-regulated to allow host cells to grow normally before induction and, after IPTG addition, the polypeptide was produced in an insoluble form as inclusion bodies.

Inclusion bodies were harvested from *E. coli* lysates by low-speed centrifugation (12000 g) and washed with 4 M urea and deoxycholate to remove several contaminating proteins by solubilization. The BPI'-containing residue was then dissolved in 8 M urea at pH 6.0 and purified further by cation-exchange chromatography. Initial folding experiments showed that BPI' remained soluble at pH 4.0 when the urea was removed by dialysis if 2-mercaptoethanol was present. However, attempts to remove the 2-mercaptoethanol invariably resulted in reprecipitation of the protein. Subsequently, we found that inclusion of 0.5 M NaCl with the 8 M urea during solubilization caused BPI' to remain fully soluble on removal of the urea by dialysis, even without 2-mercaptoethanol. The efficiency of folding of BPI' isolated from *E. coli* by this procedure was about 70%, as measured by densitometer scanning of SDS/polyacrylamide gels containing samples from soluble and insoluble fractions. Additionally, the yield of the purified active protein was approx. 0.5–0.7 mg/l of culture. The BPI'/C3 protein was also purified to homogeneity using the purification procedure and was obtained in similar yield. Figure 2 summarizes the steps in the purification of the BPI' and BPI'/C3 proteins.

Figure 3 Antibacterial activity of the BPI' and BPI'/C3 proteins

S. typhimurium SL761 cells were incubated with increasing amounts of BPI' (■) or BPI'/C3 (▲), plated on LB plates and incubated overnight. The resulting colonies were scored and the percentage viability was determined relative to the number of colonies obtained with untreated cells.

Table 1 Effect of boiling or incubation with trypsin or LPS on the bactericidal activity of BPI' and BPI'/C3

Where relevant 4 μg of BPI' or BPI'/C3 in 20 mM phosphate buffer, pH 6.0, was added to 1×10^6 SL761 cells. In some cases the recombinant proteins were boiled for 10 min immediately before additions were made. Alternatively, the proteins were pre-incubated with trypsin (100 μg) or *E. coli* R_a LPS (4 μg). Data represent the means of at least three very similar experiments.

Addition	Viability of SL761 cells (%)
None	100
BPI'	0.5
BPI'/C3	1.3
Boiled BPI'	6.02
Boiled BPI'/C3	5.3
Trypsin-treated BPI'	96.82
Trypsin-treated BPI'/C3	135.4
LPS-treated BPI'	102.34
LPS-treated BPI'/C3	136.6

N-terminal sequence determination of the BPI' protein

Sequential Edman degradation of the BPI' protein gave the sequence H₂N-Met-Val-Asn-Pro-Gly-Val, showing that the initiating formyl-methionine residue was not removed following induction of synthesis of the polypeptide.

Antibacterial activity of BPI' and BPI'/C3

Incubation of *E. coli* C600 or *S. typhimurium* SL761 with BPI' reduced cell viability in a dose-dependent manner with an LD₅₀ of 0.3 μg for 1×10^6 SL761 cells (Figure 3). Surprisingly, the BPI'/C3 derivative also displayed potent bactericidal activity, implying that insertion of polio virus epitope between residues

Figure 4 Urea denaturation of BPI'/C3 as monitored by fluorescence emission spectroscopy

BPI'/C3 (10 μg/ml, final concentration) was incubated with various concentrations of urea for 1 h at room temperature. The fluorescence emission spectra were then recorded at λ_{ex} 280 nm and the denaturation curve deduced from the ratio between the fluorescence intensities at 340 and 355 nm.

Ala¹¹⁵ and Asp¹¹⁶ did not affect this function of the protein. Pre-incubation of either protein with trypsin or with *E. coli* R_a LPS restored cell viability, suggesting that a protein with LPS-binding capability was indeed responsible for the observed antimicrobial activity (Table 1).

Effect of denaturation on bactericidal activity

Boiling of BPI' or BPI'/C3 proteins immediately before addition to cells did not significantly affect their antimicrobial action (Table 1). This observation suggested that the proteins might be capable of bactericidal action even when presented to cells in an unfolded form. To explore this possibility we measured the change in intrinsic fluorescence that occurred upon denaturation of BPI'/C3 *in vitro* and, additionally, the kinetics of protein renaturation.

Incubation of BPI'/C3 with increasing concentrations of urea resulted in a shift of the tryptophan fluorescence emission maximum from 340 to 355 nm, indicating that the residues were subject to a change of environment (Figure 4). A large decrease in the fluorescence intensity ratio occurred with urea addition, even with urea concentrations as low as 0.5 M, suggesting that the protein was highly susceptible to unfolding. To determine the kinetics of renaturation, denatured BPI'/C3 in 8 M urea was diluted 200-fold into citrate/phosphate buffer and the resulting change in fluorescence measured at various times. Figure 5 shows that even after incubation for 2 h the treated protein had not completely refolded, as assayed by fluorescence changes.

As the above experiments suggested that BPI'/C3 was readily unfolded *in vitro* and that considerable time was required for renaturation, the bactericidal activity of the protein in the presence of urea was measured. Although incubation of *S. typhimurium* SL761 with concentrations of urea > 1 M caused a total loss of viability, incubation with 0.5 M urea allowed 50% of the cells to survive. By contrast, when 5 μg of BPI' was added to cells under the same conditions, only 28% of cells survived (Table 2), indicating that the protein had significant bactericidal activity under these conditions.

Figure 5 Kinetics of refolding of BPI'/C3 as measured by the intrinsic fluorescence of tryptophan residues

BPI'/C3 was denatured in 8 M urea and diluted 200-fold into 50 mM citrate/phosphate buffer (pH 4.0). The fluorescence intensity at 355 nm (λ_{ex} 280 nm) was recorded at various times after dilution. The triangles and squares correspond to native and urea-treated BPI'/C3 protein respectively.

Table 2 Effect of urea-treated BPI' on viability of SL761 cells

Present in incubation mix	Survival (%)
SL761	100.0
SL761 + BPI'*	9.3
SL761 + urea†	52.0
SL761 + urea + BPI'	27.6

* The SL761 cells were incubated with 4.0 mg of BPI' for 30 min at 37 °C; other conditions were as described in the Materials and methods section.

† The concentration of urea used was 0.5 M.

Previous studies indicate that the ultimate cellular target for BPI action is the cytoplasmic membrane [7,12]. Accordingly, one interpretation of these results is that BPI' may unfold, either partially or completely, to translocate across the cell envelope. However, the possibility that BPI' or BPI'/C3 refolding is much more rapid in the presence of cells cannot yet be ruled out and therefore further investigation is required.

Activation of a specific phospholipase A₂ by BPI'

Native BPI activates the hydrolysis of phospholipids by certain phospholipases A₂ and it has been established that this effect correlates with the presence of positively charged amino acids in the N-terminal region of the enzyme, including residues 6, 7, 10, 11 and 15 [11]. Inspection of the recently determined sequence of the phospholipase A₂ enriched in rheumatoid arthritic synovial fluid strongly suggested that this enzyme should also be BPI-responsive [29]. Figure 6 shows that phospholipid hydrolysis by the synovial fluid phospholipase was indeed stimulated by native BPI as well as by BPI'. When corrected for the smaller size of the latter protein, BPI and BPI' caused 7.2-fold and 2.5-fold increases in phospholipid hydrolysis respectively, with 10 ng of the enzyme.

Figure 6 Selective activation of phospholipid hydrolysis due to a synovial fluid phospholipase A₂ (PLA₂) by the BPI' and native BPI proteins

A recombinant form of human synovial fluid PLA₂ or pig pancreatic PLA₂ was added to *E. coli* CE1303 cells, previously labelled with [¹⁴C]oleic acid, in the presence or absence of BPI' or native BPI. In each case 1 µg of BPI or BPI' protein was added to the standard assay mixture. The [¹⁴C]-labelled fatty acids released upon phospholipid hydrolysis were measured as described previously [25]. ★, synovial fluid PLA₂ with human BPI; ◆, synovial fluid PLA₂ with BPI'; ▲, pig pancreatic PLA₂ with BPI; ▼, synovial fluid PLA₂ alone; ■, pig pancreatic PLA₂ alone. Data represent the means of at least two very similar experiments.

Table 3 Increase in outer membrane permeability to actinomycin D mediated by BPI' protein

1×10^8 *E. coli* or *S. typhimurium* cells were pre-incubated with BPI', BPI'/C3 or buffer. Actinomycin D and TRANS ³⁵S-LABEL (a mixture of [³⁵S]Cys and [³⁵S]Met) were then added and, after further incubation, trichloroacetic acid-precipitable material was collected and counted.

Bacterial strain	Protein added	Incorporation of radioactivity (%)
C600	None	100.0
C600	BPI'	16.9
C600	BPI'/C3	21.3
SL761	None	100.0
SL761	BPI'	6.8
SL761	BPI'/C3	8.2

As expected, neither protein had any effect on the activity of pig pancreatic phospholipase A₂, even using up to 400 ng of the enzyme.

Permeabilization of the outer membrane

Actinomycin D usually has no significant effect on *E. coli* and *S. typhimurium* cells as, like many hydrophobic compounds and larger hydrophilic molecules [30], it cannot penetrate the outer membrane. By contrast, incubation of *E. coli* cells with the antibiotic in the presence of BPI' for 30 min resulted in inhibition of the incorporation of cysteine and methionine into polypeptides, as measured by the decrease in the incorporation of the ³⁵S-labelled amino acids into trichloroacetic acid-precipitable material (Table 3). It was therefore concluded that, like BPI, the recombinant protein increases the permeability of the outer membrane to compounds such as actinomycin D that are

Figure 7 (a) Binding of BPI' and BPI'/C3 to *S. typhimurium* and (b) a Scatchard plot of the BPI' binding data

(a) [^{35}S]Methionine-labelled BPI' (■) or BPI'/C3 (▲) was added to *S. typhimurium* SL761 (1×10^7 cells) and incubated for 30 min at 37 °C before washing and counting of bound label (see Materials and methods section). (b) A similar analysis was performed on the BPI'/C3 data to obtain the apparent dissociation constants for both proteins.

normally excluded from the cell. Similar results were obtained with the BPI'/C3 derivative (Table 3).

Affinity of the BPI' and BPI'/C3 proteins for *E. coli* cells

Native BPI has a single high-affinity binding site on the *E. coli* cell surface with an apparent K_d of 23 nM [12]. To obtain quantitative data on the affinities of the truncated (and non-glycosylated) BPI' and BPI'/C3 derivatives, the proteins were labelled *in vivo* with [^{35}S]methionine, purified to homogeneity and tested for binding to *S. typhimurium* SL761 cells (Figure 7). BPI' showed saturation binding of 1×10^7 cells at 1.8 μg (equivalent to 3.88×10^6 sites per cell) whereas 23 μg of BPI'/C3 was required to achieve the same degree of binding (equivalent to

4.84×10^6 sites per cell). Moreover, Scatchard analyses indicated that the BPI' and BPI'/C3 proteins have a single type of site on SL761 cells with apparent K_d values of 36 and 74 nM respectively. Unlike native BPI, the recombinant proteins were not efficiently released following the addition of high concentrations of Mg^{2+} ions. Only 12% and 19% of the bound BPI' and BPI'/C3 protein respectively, dissociated following the addition of 200 mM MgCl_2 , whereas 90% of native BPI is released with 40 mM MgCl_2 . Assay of eluted protein by densitometry of Coomassie-Blue-stained gels gave similar results (data not shown). We conclude that, once bound, the truncated, non-glycosylated derivatives of BPI display extremely stable interactions with the bacterial cell surface.

DISCUSSION

BPI has a remarkable range of diverse biological effects on Gram-negative bacteria, only some of which are likely to be directly involved in its bactericidal activity [3,12]. Dissection of the functions of BPI has been hampered by the relatively small amounts of the native protein that can be obtained from leucocytes and therefore the types of experimental approaches that can be used. In this paper we describe the design and construction of a synthetic gene, based on the amino acid sequence of human BPI, and the characterization of the resulting gene product. By chemically synthesizing the gene it has been possible to design a DNA sequence with several novel features that facilitate subsequent studies. For example, codon usage has been adjusted to optimize gene expression in *E. coli* and unique restriction enzyme sites have been incorporated into the coding region to aid mutagenesis of key segments.

Given the potent antimicrobial activity of the native protein, it was anticipated that it might prove difficult to clone the BPI' gene in *E. coli*. Indeed, the gene was highly unstable in several commonly used vectors (S.-Y. Qi and C. D. O'Connor, unpublished work). By contrast, expression in vector pET11 was sufficiently well-regulated to allow the host cells to grow to high densities before induction of synthesis of BPI'. After induction, the protein was produced in an aggregated form that presumably accounts for its lack of biological activity on host cells at this stage. Characterization of the recombinant protein obtained after purification and refolding indicated that the cytotoxic and permeability-increasing activities of BPI were retained (Figure 3; Tables 1 and 3), ruling out the possibility that glycosylation is required for these functions. Further, BPI' selectively stimulated phospholipid hydrolysis by a synovial fluid phospholipase A_2 (Figure 6), establishing for the first time that this property of BPI is also carried in the N-terminal half of the protein. Recent work suggests that the BPI'/C3 protein also retains this property of native BPI (Y. Li, S.-Y. Qi, D. C. Wilton and C. D. O'Connor, unpublished work).

Quantitative binding studies indicated that BPI' bound with high affinity to bacteria (apparent K_d 36 nM). Additionally, the BPI'/C3 derivative also bound strongly, although it had a higher apparent K_d (74 nM). The data obtained are similar to those reported by Mannion et al. [9] for the native human protein. However, it should be noted that the previous study employed an *E. coli* R_c strain (rather than the *S. typhimurium* strain used here) and binding measurements were carried out at pH 6.0 rather than at pH 4.0. Hence, our results are not directly comparable. In contrast with the native protein, BPI' was not readily dissociated from cells with high concentrations of Mg^{2+} . Less than 20% was eluted with 200 mM MgCl_2 , whereas about 90% of native BPI dissociated under the same conditions. Consequently, it appears that, once bound, the recombinant protein is

very stably associated with its LPS receptor. The native protein is likely to be susceptible to degradation in its natural setting (the neutrophil phagosome) due to the presence of multiple proteases. It is therefore possible that similar truncated BPI-LPS complexes are produced normally and that they serve to sequester LPS following the disintegration of the bacterial cell, thereby preventing undesirable endotoxic effects.

Studies by Elsbach, Weiss and colleagues have established that disruption of the outer membrane by BPI (e.g. increased permeability to hydrophobic agents) can be reversed by trypsin treatment or the addition of bivalent cations [10]. Moreover, it is possible to isolate mutants of *E. coli* and *S. typhimurium* that show similar increases in outer membrane permeability and yet grow normally [31,32]. These findings suggest that damage to the outer membrane is not primarily responsible for the bactericidal activity of BPI. Indeed, recent work indicates that the principal site affected is the cytoplasmic membrane [7,12]. While BPI may cause these effects via some intermediate, an attractive possibility is that BPI molecules traverse the bacterial cell envelope to act directly on some component of the cytoplasmic membrane. This obviously raises the question of how BPI might penetrate the outer membrane. The finding that apparently denatured BPI' protein retains bactericidal activity may be significant in this respect, as it suggests that the polypeptide may unfold to penetrate the membrane. Interestingly, strikingly similar results have recently been reported by Benedetti et al. for colicin A, an apparently unrelated bactericidal toxin that is known to act on the cytoplasmic membrane [33]. Indeed, the fluorescence and potassium efflux assays used by this group suggest that a partial unfolding of the colicin A polypeptide may actually accelerate its translocation through the *E. coli* cell envelope. In the case of BPI we cannot rule out the possibility that the rate of refolding is increased in the presence of cells. Consequently, further studies are needed to prove conclusively that BPI unfolds and penetrates the outer membrane.

The utility of the synthetic gene for mutagenesis has been highlighted by results with the BPI'/C3 derivative, which retained the bactericidal and membrane-perturbing properties of the parent protein. As pre-incubation of the protein with LPS blocked its bactericidal action, it is also likely that the BPI'/C3 protein has LPS-binding activity. Because the protein does not appear to differ in any essential way from BPI', the region containing the inserted epitope cannot be directly involved in any of the major activities of BPI. Moreover, insertion of the epitope does not appear to have caused gross disruption of the structure of the protein (as the derivative was functional), suggesting that the region in question is at the surface of the protein rather than buried in its interior. The availability of functional recombinant derivatives of the BPI protein paves the way for detailed structural and biochemical studies. In particular, it should now be possible to alter key residues to assess their involvement in LPS binding or in the bactericidal activity of the protein.

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