Functional Domains of Recombinant Bactericidal/Permeability Increasing Protein (rBPI23)*

(Received for publication, August 12, 1993)

Roger G. Little†§, Drew N. Kelner†, Edward Lim†, David J. Burke§ and Paul J. Conlon†‡‡

From the Departments of †Immunology and ‡Analytical Biochemistry, XOMA Corporation, Berkeley, California 94710

The 28-kDa recombinant amino-terminal bactericidal/permeability increasing protein fragment (rBPI28) has all of the antibacterial and antiendotoxin properties of the holoprotein. In the current studies, we have identified multiple active domains within rBPI28 with chemical and proteolytic cleavage fragments and with synthetic overlapping peptides. We also demonstrate a novel, high affinity heparin binding property for rBPI28, in addition to its established bactericidal and lipopolysaccharide binding properties.

Cleavage fragments and synthetic, overlapping peptides of rBPI28 were analyzed for inhibition of the lipopolysaccharide-induced Limulus amebocyte lysate reaction, for bactericidal activity, and for heparin binding. Three separate, active domains were identified in amino acid regions 17-45, 65-99, and 142-169. A single synthetic peptide (85-99) was bactericidal. These results indicate that rBPI28 is comprised of three separate functional domains which contribute to the high affinity interaction of rBPI28 with Gram-negative bacteria. The individual activity of each domain and the cooperative interaction among domains provide the basis for developing rBPI28 analogues with increased biologic efficacy.

Bactericidal/permeability increasing protein (BPI) is a 55-kDa cationic protein present in the azurophilic granules of neutrophils. BPI is specifically bactericidal to Gram-negative organisms due, in part, to its initial high affinity binding to the outer cell membrane lipopolysaccharide (LPS) (2), followed by bacterial killing via an oxygen independent mechanism (3, 4). Recently, BPI has been shown to bind to the structurally conserved lipid A region of LPS (5). This high affinity interaction of BPI with lipid A also results in inhibition of LPS-dependent biologic responses (6).

Interestingly, a proteolytically derived 25-kDa amino-terminal fragment of the native BPI has all of the antibacterial and antiendotoxin properties of the whole molecule (6-8). A recombinant 25-kDa amino-terminal fragment of BPI (rBPI25) also has equivalent or greater activity than the holoprotein in bactericidal and LPS binding assays (5,8). The carboxyl-terminal half of the protein may be involved in localizing the molecule within the neutrophil granule, but its precise function is unknown (6,9).

Functional domains may exist within the 25-kDa amino-terminal fragment of BPI. Many bactericidal proteins share the common structural motif of an amphipathic α helix characterized by a cationic region which is opposed by a hydrophobic region. Bactericidal sites can be formed by a continuous amino acid sequence or by discontinuous sequences that are brought together by the three-dimensional structure of the molecule (10). For example, a continuous bactericidal site is found at the amino terminus of the cecropins, a family of antibacterial peptides found in the hemolymph of lepidopteran insects (11). Alternatively, a discontinuous bactericidal site can be found in the betacencins from bovine neutrophils (12) and in the defensins (13). To date, the bactericidal region(s) within BPI have not been described.

Many mammalian proteins isolated from blood bind to soluble heparin or cell surface heparan with a heparin binding domain characterized by a cluster of basic and hydrophobic amino acids (14). This similarity of the heparin binding motifs to the bactericidal/LPS binding motifs prompted us to analyze the potential heparin binding by rBPI28. Heparin is a sulfated polysaccharide with a net negative charge and a molecular mass range of 6 to 25 kDa (15). In general, heparin binding proteins can be categorized into two classes. The first class consists of proteins which require heparin as a cofactor to elicit a specific biologic response. Examples of this first type of heparin binding protein include fibroblast growth factors (16, 17), endothelial cell growth factors (18), and antithrombin III (19). The second class of heparin binding proteins consists of proteins which neutralize the heparin-dependent stimuli of the first class of heparin binding proteins. Examples of this second type include platelet factor IV (20) and thrombospondin (21). Other proteins such as interleukin 3 (22), interleukin 8 (23), complement C1q (24), granulocyte/macrophage colony stimulating factor (22), and heparin binding protein (CAP37, azurocidin) (25) also bind to heparin, but the function of this binding has not been determined. Heparin may protect these proteins from proteolytic degradation and/or serve as a target for localization of proteins to heparan-rich cells (i.e. endothelial cells) or to the heparan-rich extracellular matrix.

We report herein that in addition to its bactericidal and high affinity LPS binding properties, human rBPI28 binds specifically to heparin. We also describe the fragmentation of rBPI23 by cyanogen bromide (CNBr) or by endoproteinase Asp-N digestion and subsequent analysis of the fragments for LPS interactions, bactericidal activity, and heparin binding. Overlapping peptides of the rBPI23 sequence were also synthesized and analyzed for activity to further define the functional domains of the recombinant molecule. We have identified three separate, active domains within the rBPI23 molecule that contribute to the overall biologic activity.
**rBPI13 Functional Domains**

**EXPERIMENTAL PROCEDURES**

**Reduction and Alkylation of rBPI13**—The production and purification of rBPI13 will be described in detail elsewhere. The rBPI13 was reduced and alkylated prior to proteolysis by cyanogen bromide or endoproteinase Asp-N. The protein was desalted by cold (4 °C) acetone precipitation (1:1 v/v) overnight and pelleted by centrifugation (5000 × g) for 10 min. The rBPI13 pellet was washed twice with cold acetone and dried under a stream of nitrogen. The rBPI13 was then reconstituted to 1 mg/ml in 8 M urea/0.1 M Tris, pH 8.1, and reduced by adding an addition of 3.4 M Na thiosulfate (Calbiochem) for 90 min at 37 °C. Alkylation was performed by the addition of iodoacetamide (Sigma) to a final concentration of 5.3 mM for 30 min in the dark at room temperature. The reduced and alkylated protein was acetone-precipitated and washed as described above, and the pellet was redissolved for either CNBr or Asp-N digestion.

**Cyanogen Bromide Cleavage**—Reduced and alkylated rBPI13 was dissolved in 70% trifluoroacetic acid (TFA) (protein sequencing grade, Sigma) to a final protein concentration of 5 mg/ml. Cyanogen bromide (Baker Analyzed Reagent, VWR Scientific, San Fransisco, CA) dissolved in 8 M urea/0.1 M Tris, pH 8.1, was added at a 1:1 ratio (w/w) to the protein to give a final protein concentration of 1 mg/ml. The reaction was allowed to proceed for 2 h at 37 °C. The reaction was stopped by the addition of 100 μl of 25% acetic acid. Absorbance was quantitated at 405 nm using a Vmax microplate reader (Molecular Devices, Menlo Park, CA).

**Radial Diffusion Bacterial Assay**—The rBPI13 synthetic peptides, CNBr cleavage fragments, and Asp-N digestion fragments were analyzed for inhibition of bacterial growth in a radial diffusion assay (28). Specifically, an overnight culture of E. coli J5 was diluted 1:50 into tryptic soy broth (Difco) and incubated for 3 h to midlog phase. The bacteria were centrifuged at 1500 × g for 5 min in a Sorvall RT6000GB, resuspended, and washed with 10 ml sodium phosphate buffer, pH 7.4, and finally resuspended in phosphate buffer (5 ml). Bacterial growth was determined by measuring absorbance at 590 nm (1.25 × 10⁶ colony forming units/ml yield A₅₉₀ = 1.00). The bacteria were diluted to 4 × 10⁹ colony forming units/ml in 10 ml of molten underlayer agarose. The underlayer agarose was composed of 1% agarose M (Pharmacia, Uppsala, Sweden), 3% tryptic soy broth, and 0.02% Tween 20 (Sigma) dissolved in phosphate buffer. The mixture was poured into a square, 100 × 100 × 15-mm Petri dish (Baxter Scientific Products, McGaw Park, IL), and allowed to solidify. A series of wells were punched into the agarose with a sterile 3-mm punch attached to a vacuum source. A volume of 10 μl containing rBPI13 fragments, or peptides (0.1–10 μg) was added to individual wells. The plates were incubated at 37 °C for 3 h. After incubation, the agarose was poured off on top. Overlayer agarose was composed of 1% agarose, 6% tryptic soy broth in the phosphate buffer. The overlayer was allowed to harden, and the plates were incubated overnight at 37 °C. Bacterial zones were visualized by staining the plates for 24 h with a solution of 0.002% Coomassie Brilliant Blue R-250 (Bio-Rad), 27% methanol, 5% acetic acid, and 15% formaldehyde (Sigma) in distilled water. The bacterial zones were measured with a micrometer (±0.05 mm), and data are expressed as area in mm².

**Direct Heparin Binding Assay**—A direct [3H]heparin binding assay (29) was modified by using a polyvinylidene difluoride membrane (Immobilon-P, Millipore) to bind proteins/peptides with a higher capacity than nitrocellulose (30). The hydrophobic membrane was hydrated by successively aspirating 100 μl of ethanol and then water through each well of a 96-well Multiscreen IP plate as recommended by the manufacturer.

**Direct Heparin Binding Assay**—A direct [3H]heparin binding assay (29) was modified by using a polyvinylidene difluoride membrane (Immobilon-P, Millipore) to bind proteins/peptides with a higher capacity than nitrocellulose (30). The hydrophobic membrane was hydrated by successively aspirating 100 μl of ethanol and then water through each well of a 96-well Multiscreen IP plate as recommended by the manufacturer.

**RESULTS**

**rBPI13 Fragments**

Fragmentation by Cyanogen Bromide (CNBr) and Endoproteinase Asp-N—rBPI13 contains 6 methionine residues (positions 56, 70, 100, 111, 170, and 196), and chemical cleavage by CNBr produced six major peptide fragments. The results of the CNBr cleavage experiments are summarized in Table 1. The fragments were isolated by reverse phase (C₁₈) HPLC (Fig. 1A), and their amino-terminal sequences were determined by Edman degradation. The largest fragments (C₁ and C₅) were not resolved by the C₅ HPLC column; further attempts to resolve them by ion exchange chromatography were unsuccessful, presumably because they are similar in length and isolectric point. The identities of the C₁, C₅ fragments within the mixture were determined by ESI-MS. The predicted mass of C₁ is 6269 (Table 1), taking into account the loss of 30 atomic mass units resulting from the conversion of the carboxyl-terminal...
methionine to homoserine during the CNBr cleavage reaction. The observed mass of 6251.51 ± 0.34 is consistent with the loss of a water molecule (18 atomic mass units) in a homoserine lactone intermediate, which may be favored over the formation of the homoserine because of the hydrophobicity of the Cl fragment carboxy-terminal amino acids. The predicted mass of the C5 fragment is 6487, and the observed mass is 6485.84 ± 0.39 (Table I). For the C5 fragment, the carboxyl-terminal amino acids are hydrophilic, so the hydrolysis of the homoserine lactone intermediate is probably favored. From both the amino-terminal sequencing and the mass spectra, the C5 component represents approximately 10–25% of the material in the C1/C5 mixture.

Proteolytic cleavage with endoproteinase Asp-N was performed to provide additional fragments for the regions contained within the CNBr C1/C5 mixture. There are 6 aspartic acid residues within the rBPI33 sequence (positions 15, 36, 39, 57, 105, and 116). The six major Asp-N fragments isolated by C3 HPLC (Fig. 1B) were sequenced, and masses were determined by ESI-MS (Table I). A short duration digest at a 1:1000 (w/w) enzyme:substrate ratio was used to eliminate potential nonspecific cleavages, particularly at glutamic acid. It is evident that this digestion did not proceed to completion, as one fragment (1–38) was isolated where Asp residues’ amino acids 15 and 35 were not cleaved. The mass spectra of the Asp-N fragments were consistent with the predicted masses for each individual fragment. Unlike the CNBr cleavage, where the carboxy-terminal fragment was poorly resolved, the Asp-N fragment from amino acid 116 to the carboxyl terminus was well resolved from all of the other Asp-N fragments.

**Bactericidal Activity**—No bactericidal activity was demonstrated for the rBPI33 fragments generated by CNBr or by Asp-N digestion, when tested up to 25 pmol/well. This assay detected measurable bactericidal activity with as little as 0.75 pmol of rBPI33 per well. Reduced and alkylated rBPI33 (up to 100 pmol/well) also was not bactericidal, while alkylated rBPI33 retained bactericidal activity equivalent to rBPI33 (data not shown).

**Inhibition of the LPS-induced LAL Reaction**—BPI inhibits LPS stimulation of the Limulus amebocyte lyse proteolytic cascade (31). The results shown in Fig. 2 demonstrate that rBPI33 fragments from both the CNBr and the Asp-N digests also significantly inhibit the LPS-induced LAL reaction. The CNBr digest fraction containing amino acid fragments 1–56 and 112–170 (C1/C5) inhibited the LPS-induced LAL reaction with an IC50 of approximately 100 nm. This IC50 is approximately 10-fold higher than the IC50 for rBPI33 (9 nm) in the same assay. CNBr digest fragments not shown in Fig. 2 were noninhibitory.

A slightly different result was observed with fragments generated from the Asp-N digest, where three fragments were found to be inhibitory in the LAL assay (Fig. 2). The fragment corresponding to amino acids 116–193 (A6a) exhibited LAL inhibitory activity similar to intact rBPI33 with complete inhibition of the LPS-induced LAL reaction at 15 nm. The fragments corresponding to amino acids 57–104 (A4) and 1–38 (A1) also inhibited the LAL assay, but required 10-fold higher amounts. These results, in combination with the CNBr digest results, indicate that at least three regions of the rBPI33 molecule have the ability to neutralize LPS activation of the LAL reaction with the most potent region appearing to exist within the 116–193 (A6a) fragment.

**Binding to Heparin**—rBPI33 bound to [3H]heparin in a direct binding assay with relatively high affinity (Kd = 70 nm) and saturable binding (Fig. 3). The binding of radiolabeled heparin was completely inhibited by a 100-fold excess of unlabeled heparin (data not shown). rBPI33 binding to heparin was also demonstrated by heparin affinity chromatography. Bound rBPI33 was eluted during a linear NaCl gradient (0.15 to 2.0 M NaCl) with a single peak at 0.84 M NaCl (data not shown).

Binding of rBPI33 CNBr fragments to heparin was estimated using 100 pmol of each fragment per well with a saturating concentration of [3H]heparin (20 μg/ml). The results (Table II) indicate that CNBr fragments containing the amino acids 71–100 (C3) and 1–56 and 112–170 (C1/C5) bound heparin to a similar extent. The CNBr fragment 171–193 had lower heparin binding than C3 or C1/C5. Each of the above fragments bound more heparin than the control protein, thaumatin, a protein of similar molecular weight and charge to rBPI33.

Three Asp-N rBPI33 fragments also demonstrated heparin binding. As seen in Table II, the 57–104 Asp-N fragment (A4) bound the highest amount of heparin, followed by the 1–38 (A1A2) and 116–193 (A6a) fragments. These data, in combination with the CNBr fragment data, indicate that there are at least three separate heparin binding regions within rBPI33 with the highest capacity residing within residues 71–100.

**Synthetic 15-mer rBPI33 Peptides**

The above results suggested that at least three regions of rBPI33 contained LPS neutralization and heparin binding ac-

### Table I: Summary of rBPI33 cleavage fragment analysis

| Peak | Sequence* | Identifier sequence | Measured Mass | Predicted Mass |
|------|-----------|---------------------|---------------|---------------|
| CNBr cleavage fragmentsb | | | | |
| I | 101–110 | C4 (101–111) | ND* | 1169 |
| II | 57–67 | C2 (57–70) | ND | 1661 |
| III | 71–99 | C3 (71–100) | ND | 3404 |
| IV | 171–194 | C6 (171–196) | ND | 2929 |
| V | 1–25, 112–124 | C1 (1–56), C5 (112–170) | 6251.5 | 6269 |
| 6485.8 | 6487 |
| Asp-N proteolytic fragmentsb | A | 1–14 | A1 (1–14) | 1465.6 |
| I | 39–56 | A3 (39–56) | 2145.2 |
| II | 15–38 | A2 (15–38) | 2723.5 |
| III | 57–76 | A4 (57–76) | 5442.5 |
| IV | 1–30 | A1A2 (1–38) | 4171.4 |
| VI | 116–134 | A6a (116–193) | 8800.3 |
| VII | 116–128 | A6b (116–195) | 8997.1 |

* Sequence was identified by amino-terminal protein sequencing.

b Fragments were purified by reverse-phase HPLC as described under "Experimental Procedures" and analyzed by ESI-MS for determination of molecular mass.

ND, not determined.
FIG. 1. HPLC analyses of cyanogen bromide and endoproteinase Asp-N digests. High performance liquid chromatograms of crude cyanogen bromide digest (A) and endoproteinase Asp-N digest (B). A 50-µl sample of each digest was subjected to reversed-phase chromatography on a Zorbax Protein Plus C_{30} column (4.6 x 250 mm, 300-A pore size). Mobile phase A was 5% acetonitrile/0.1% TFA; mobile phase B was 80% acetonitrile/0.065% TFA. For the cyanogen bromide digests, the following gradient was used: 0% B for 5 min, 0–25% B in 34 min, hold for 8 min, 35–42% B in 35 min, 42–100% B in 5 min, hold at 100% B for 2 min, 100–0% B in 2 min, and re-equilibration for 46 min. For the endopeptinase
**Fig. 2. BPI fragment inhibition of LPS-induced stimulation of the Limulus ameocyte lysate.** Varying concentrations of rBPI\textsubscript{23} and rBPI\textsubscript{23} cleavage fragments were added to the LAL reaction to LPS as described under "Experimental Procedures." Absorbance was quantitated at 405 nm, and data are expressed as the mean of triplicate samples ± S.D. for each concentration.

**Fig. 3. Saturation curve of the heparin binding to rBPI\textsubscript{23}.** Specific [\textsuperscript{3}H]heparin binding was determined as described under "Experimental Procedures," and data are expressed as the mean of duplicate wells ± the range between the two values. The apparent affinity constant ($K_d = 70$ nM, assuming average heparin molecular mass = 15,000 daltons) was determined by nonlinear curve-fitting of the affinity constant ($K_d = 70$ nM, assuming average heparin molecular data using a one-site model with GraFit software, Erithacus Products Ltd.

**Activity.** To examine the rBPI\textsubscript{23} sequence in greater detail, overlapping 15-mer peptides were synthesized and tested. Samples of 10 peptides chosen randomly were analyzed by reverse phase HPLC and found to contain one major peak accounting for more than 85% of the peak areas in the sample (data not shown).

**Bactericidal Activity.** Bactericidal assays on the synthetic rBPI\textsubscript{23} peptides indicated that only one peptide (85–99) was bactericidal (Fig. 4A). This same sequence is present in the CNBr fragment 71–100 (C3) and in the Asp-N fragment 57–104 (A4).

**TABLE II**

| Fragments | Region         | [\textsuperscript{3}H]Heparin bound (cpm) |
|-----------|----------------|------------------------------------------|
| CNBr digest |                |                                          |
| C1, C5    | 1–56, 112–170  | 82,918 ± 4,462                           |
| C2        | 57–70          | 6,262 ± 182                              |
| C3        | 71–100         | 81,655 ± 3,163                           |
| C4        | 101–111        | 4,686 ± 4                               |
| C6        | 171–196        | 26,204 ± 844                             |
| Asp-N digest |                |                                          |
| A1A2      | 1–38           | 17,002 ± 479                             |
| A2        | 15–38          | 3,042 ± 162                              |
| A3        | 39–56          | 8,664 ± 128                              |
| A4        | 57–104         | 31,155 ± 1,085                           |
| A6a       | 116–193        | 33,419 ± 309                             |
| rBPI\textsubscript{23} | 1–193          | 51,222 ± 1,808                           |
| Thaumatin |                | 7,432 ± 60                               |
| Wash buffer |              | 6,966 ± 46                               |

**Inhibition of LPS-induced LAL Reaction.** Three regions of overlapping synthetic rBPI\textsubscript{23} peptides inhibited the LPS-induced LAL reaction (Fig. 4B). The most active domain resides within residues 73–99. A second active region was seen between residues 140 and 170 and a third between residues 25 and 50. A possible fourth minor domain was noted in the region, 118–128. These domains correspond to similar regions identified with the rBPI\textsubscript{23} cleavage fragments.

**Binding to Heparin.** Three major domains of rBPI\textsubscript{23} synthetic peptides also bound heparin (Fig. 4C). The most active heparin-binding region encompassed residues 73–99. A second active region was evident from residues 25–50 and a third

Asp-N digests, the gradient was as follows: 0% B for 5 min, 0–20% B in 25 min, 20–50% B in 110 min, 50–100% B in 5 min, 100–0% B in 2 min, and re-equilibration for 33 min. The flow rate was 0.2 ml/min, and absorbance was monitored at 220 nm. **Dashed line** indicates percent B for the duration of the gradient.
region appeared between residues 140 and 170. There was a significant correlation ($r = 0.75$, $p < 0.0001$, $n = 47$) between heparin binding capacity and percent inhibition of the LPS-induced LAL activity for each peptide.

**DISCUSSION**

Overall, our results indicate that rBPIZ3 contains three functional domains that contribute to the total biological activity of
The molecule (Fig. 5). The first domain appears in the sequence of amino acids 17-45 and is destroyed by Asp-N cleavage at residue 38. This domain is moderately active in both the inhibition of LPS-induced LAL activity and in heparin binding assays. The second active domain appears in the region of amino acids 65-99, and its inhibition of LPS-induced LAL activity is diminished by CNBr cleavage at residue 70. This domain also exhibits the highest heparin binding capacity and contains the bactericidal peptide, 85-99. The third active domain, between amino acid residues 142 and 169, inhibits LPS-induced LAL activity and exhibits the lowest heparin binding capacity of the three regions.

The lipo-oligosaccharide (apparent of LPS, and increased O-antigen polysaccharide chain length, between amino acid residues neutralize it, albeit with lower affinity

The molecule (Fig. 5). The first domain appears in the sequence of amino acids 17-45 and is destroyed by Asp-N cleavage at residue 38. This domain is moderately active in both the inhibition of LPS-induced LAL activity and in heparin binding assays. The second active domain appears in the region of amino acids 65-99, and its inhibition of LPS-induced LAL activity is diminished by CNBr cleavage at residue 70. This domain also exhibits the highest heparin binding capacity and contains the bactericidal peptide, 85-99. The third active domain, between amino acid residues 142 and 169, inhibits LPS-induced LAL activity and exhibits the lowest heparin binding capacity of the three regions.

rBPIz3 specifically interacts with LPS with high affinity binding (apparent \( K_d = 2-5 \text{ nM} \)) to the conserved lipid A moiety of LPS, and increased O-antigen polysaccharide chain length does not diminish the apparent \( K_d \) (5). The lipid A of LPS is responsible for the majority of the toxic and other biological effects of LPS (32). Lipid A consists of a glucosamine dimer to which fatty acyl chains are linked through amide and ester bonds. The disaccharide carries two phosphoryl groups, one ester-linked to the nonreducing glucosamine and the other glucosidically linked to the reducing glucosamine (33). The resulting lipid A molecule is a highly amphipathic structure comprised of the hydrophobic acyl chains paired with the ionic phospholipid groups of the disaccharide. A high affinity interaction with such a molecule might require interaction with both of these moieties.

Three other well characterized proteins and peptides have been shown to interact specifically with lipid A. These proteins include polymyxin B1 (34), Limulus antilipopolysaccharide factor (LALF) (35), and LPS-binding protein (36). Polymyxin B1 is a cyclic, cationic lipopeptide from Bacillus polymyxa comprised of six \( \alpha,\gamma \)-diaminobutyric acid residues, one \( \delta \)-phenylalanine, one \( \delta \)-leucine, one \( \delta \)-threonine, and a 6-methyloctanoyl moiety (34). The combination of cationic and hydrophobic functional groups in the polymyxins is hypothesized to result in their apparent high affinity \( (K_d = 10^{-7} \text{ M}) \) for LPS (37). The polymyxins also exhibit potent bactericidal activity as a result of their ability to bind to the outer membrane LPS of Gram-negative bacteria and subsequently disrupt the inner membrane causing cytoplasmic leakage and death (37). Polymyxin analogues lacking the fatty acid moiety are still able to bind LPS and neutralize it, albeit with lower affinity \( (K_d = 1.5 \times 10^{-6} \text{ M}) \), but the bactericidal activity of these analogues is lost (38). A similar lack of bactericidal activity in synthetic polymyxin peptides without the fatty acid moiety has recently been confirmed using peptide analogues of polymyxins that were cyclized by oxidation of amino- and carboxyl-terminal cysteines (39). The LPS binding regions of LALF and LPS binding protein have not yet been elucidated.

The results from our fragmentation experiments suggest that there are three rBPIz3 domains capable of interacting with LPS. The most active cleavage fragment is found in the carboxyl-terminal region of rBPIz3 (amino acids 116-193). This region is potent in the inhibition of LPS-induced LAL activity (Fig. 2). This region includes the most hydrophobic region of rBPIz3 (amino acids 151-160) as well as all three of the cysteines in rBPIz3 (amino acids 132, 135, and 175). The hydrophobic domain (amino acids 151-160) is flanked by short sequences of cationic lysines and/or histidines. It is intriguing to postulate an intramolecular disulfide bond between two of the three cysteines (amino acids 132, 135, and 175) that would create an LPS binding loop within the rBPIz3 molecule in a fashion analogous to the discontinuous LPS binding sites of the bactenecin (12) and defensin molecules (13). The other two regions that inhibit the LAL reaction to LPS are fragments 1-38 and 57-104. The decreased inhibition of the LAL reaction to LPS by these regions may be similar to the LPS binding by the linear polymyxin analogues (38, 39).

The results from the synthetic 15-mer rBPIz3 peptides also identify three separate, continuous domains which inhibited the LPS-induced LAL reaction. Clearly, the most inhibitory domain was between residues 73 and 99. Based on the assumption that all peptides were synthesized with approximately the same yield (supported by random HPLC analysis), the most active peptide is that containing amino acids 85-99. Further studies with larger amounts of synthetic peptides from these regions will determine the relative affinities of the three regions in competition for LPS binding assays.

Bactericidal activity was observed only in the synthetic peptide containing amino acids 85-99. The lack of bactericidal activity in the CNBr and Asp-N fragments containing residues 85-99 may be due to some alteration of the secondary structure caused by amino acids beyond the 85-99 sequence. Alternatively, some alteration during reduction/alkylation or concentration effects may have caused the lack of bactericidal activity. These fragments inhibit the LAL reaction to LPS, but the bactericidal activity is greatly diminished, if not abolished. Clearly, the synthetic peptide of residues 85-99 does have bactericidal activity (albeit at higher concentrations than rBPIz3), and it is intriguing to postulate that this domain is responsible for the bactericidal properties of rBPIz3.

The bactericidal peptides, cecropins and magainins, are characterized by a continuous, amphipathic, \( \alpha \)-helical region which is necessary for activity (10, 40). The continuous amphipathic motif is located at the amino-terminal 10 amino acids of the cecropins (41). The bactericidal rBPIz3 peptide has high homology with the most active cecropin bactericidal sequence: cecropin A, K-W-K-L-F-K-K-L-E-K (1-10); rBPIz3, K-W-K-A-Q-K-R-F-L-K (90-99). The two other functional rBPIz3 domains may give the whole rBPIz3 molecule a higher affinity for the outer membrane LPS of Gram-negative bacteria than the 90-99 peptide and thus, more effectively, target this bactericidal sequence to the bacterium. Identification of this bactericidal sequence within rBPIz3 allows for future rBPIz3 analogues with increased biologic efficacy.

We observed a high degree of structural similarity between the cationic/hydrophobic motif of LPS binding/bactericidal molecules and the known consensus sequences of heparin binding proteins (X-B-B-X-B-X or X-B-B-B-X-X-B, where X is any hydrophobic amino acid and B is any basic amino acid) (14). rBPIz3 binding to soluble heparin in the direct binding assay and to solid phase heparin in the heparin chromatog-
rBPI23 Functional Domains

raphy, indicates that the rBPI23/heparin interaction is similar to other heparin binding proteins, including: IL-3, GM-CSF, IL-8, FF4, FGF-1, FGF-2, C1q, ATIII, and thrombospordin (22, 23, 20, 16, 29, 19, 21). All of these proteins, except thrombospordin, bind to heparin with one of the above cationic/hydrophobic amino acid repeating motifs (14). Thrombospordin is a heparin binding protein that requires a Trp-Ser-Trp consensus sequence for heparin interactions (21). Thus, two different types of amino acid motifs are found in heparin-binding proteins. All three active domains of rBPI23 contain aspects of both types of motifs (Fig. 5).

An unexpected finding from our data is that an excellent correlation exists between the synthetic rBPI23 peptides that bind to heparin and those which inhibit the LPS-induced LAL reaction (r = 0.75, p = 0.0001, n = 47). These data suggest that LPS and heparin may present similar charged arrays to the proteins with which they interact. Heparin is a highly sulfated polysaccharide with a molecular mass of 6 to 25 kDa and is composed of polymers of D-glucosamine-6-sulfate and N-glucosamine-N-sulfonic acid. The majority of heparin polymers consist of 8-12 disaccharide units. The overall net negative charge of heparin is due to its high content of sulfate and carboxylic acid functional groups. Sulfate esters are present at position 6 of glucosamine and position 2 of iduronic acid. Sulfamides are present at position 2 of glucosamine (15). Thus, in aqueous solution, both LPS and heparin display ionic arrays of phosphates or sulfates on a saccharide backbone. As a result, it is tempting to speculate that other proteins (e.g., polymyxins, cecropins, magainins, etc.) which bind LPS avidly may also bind tightly to heparin. Conversely, proteins which bind tightly to heparin (e.g., rBPI23) may also interact with LPS. Such interactions may lead to new clinical indications for LPS/heparin binding proteins.

Heparin is released from mast cell granules, and heparan, a cell-associated form, is found with a nearly ubiquitous distribution on mammalian cell surfaces and in the extracellular matrix. Future studies will determine the physiological role of rBPI23 heparin/heparan binding. Possible roles include protection from proteolytic degradation and/or tethering to the extracellular matrix as for FGF-2 (25). In conclusion, we have identified three separate, active domains within the rBPI23 molecule by two different techniques. One technique utilizes CNBr and endoproteinase Asp-N-derived fragments to dissect the whole molecule. The other approach uses synthetic overlapping 15-mer peptides of the rBPI23 amino acid sequence to determine the active domains. Overall, the two approaches complement each other with respect to heparin binding and LAL inhibition. Only one synthetic peptide (amino acids 85-99) was bactericidal. The contribution of all three sites, in concert, may synergize to create the total bioactivity of the rBPI23 molecule. These studies provide the rationale for the design of future rBPI23 analogues with increased biologic efficacy.

Acknowledgments—We thank Dr. Francis Bitsch and John Kim in the laboratory of Dr. Cedric Shackleton of the Children's Hospital-Oakland Research Institute for their expert mass spectrometry analysis of the BPI cleavage fragments. We are also thankful for the critical reading of this manuscript by Dr. Patrick J. Scannon, Dr. Patrick W. Trow, and Dr. Stephen F. Carroll. The expert technical assistance of J. Keith Duncan, Nancy M. Raja, and Jody W. Rosen is greatly appreciated.

Note Added in Proof—Since the submission of this manuscript, the crystal structure for LALF has been published. An amphiopathic loop structure within LALF was postulated to interact with lipopolysaccha-

REFERENCES

1. Weiss, J., Elsbach, P., Olson, I., and Odelberg, H. (1978) J. Biol. Chem. 253, 2659-2672.
2. Weiss, J., Muollo, K., Victor, M., and Elsbach, P. (1984) J. Immunol. 132, 3109-3115.
3. Elsbach, P., and Weiss, J. (1988) In: Inflammation: Basic Principles and Clinical Correlates (Galli, J. I., Goldstein, I. M., and Snyderman, R., eds) pp. 603-636, Raven Press, New York.
4. Weiss, J., Stendahl, O., and Elsbach, P. (1982) In: Advances in Experimental Medicine and Biology (Rossi, F., and Patriarca, P., eds) Vol. 141, pp. 129-137, Plenum Publishing Corp., New York.
5. Gazzano-Santoro, H., Parent, J. E., Grima, L., Horwitz, A., Parmen, T., Theofan, G., Elsbach, P., Weiss, J., and Conlon, P. J. (1992) Infect. Immun. 60, 4754-4761.
6. Ooi, C. E., Weiss, J., Doerfler, M. E., and Elsbach, P. (1991) J. Exp. Med. 174, 115-126.
7. Ooi, C. E., Weiss, J., Elsbach, P., Frangiote, R., and Mannion, B. (1987) J. Biol. Chem. 262, 14693-14694.
8. Weig, H., Elsbach, P. A., and Gewurtz, C. J., Grina, L., Horwitz, A., and Theofan, G. (1992) J. Clin. Invest. 90, 1122-1130.
9. Elsbach, P., and Weiss, J. (1993) Curr. Opin. Immunol. 5, 103-107.
10. Kopp, D. M., and Evans, R. M. (1993) J. Peptide Protein Res. 42, 277-286.
11. Steinher, A., Andreu, D., and Merrifield, R. B. (1988) Biochem. Biophys. Acta 929, 253-265.
12. Frank, R. W., Gennaro, R., Schonider, K., Przybylski, M., and Romeo, D. (1990) J. Biol. Chem. 265, 18871-18874.
13. Lehrer, R. I., Ganz, T., and Selsted, M. E. (1991) Cell 64, 229-230.
14. Carlson, A. D., and Weintraub, H. J. B. (1989) Arteriosclerosis 9, 21-32.
15. Roden, L. (1980) In: The Biochemistry of Glycoproteins and Proteoglycans (Lenzner, W. J., ed) pp. 267-371, Plenum Publishing Corp., New York.
16. Thomas, K. A., Rice-Cotton, M., and Fitzpatrick, S. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 357-361.
17. Yason, A., Klagesmun, E., Esso, J. D., Leder, P., and Ornitz, D. M. (1991) Mol. Cell. Biol. 11, 240-247.
18. Maciag, T., Mehlman, T., Friesel, R., and Schreiber, A. B. (1984) Science 225, 932-935.
19. Bjork, I., Ylenjarvi, K., Olson, S. T., and Bock, P. E. (1992) J. Biol. Chem. 267, 1976-1982.
20. Lane, D. A., Denton, J., Flynn, A. M., Thunberg, L., and Lindahl, U. (1984) Biochem. J. 218, 725-727.
21. Tarabotelli, G., Roberts, D. D., Liotta, L. A., and Giavazzi, R. (1990) J. Cell Biol. 111, 765-772.
22. Roberts, R., Gallagher, J., Spooner, E. T. D. A., Bloomfield, F., and Deter, T. M. (1988) Nature 335, 376-378.
23. Oppenheim, J. J., Zachariae, C., Mukaida, N., and Matsuhashi, K. (1991) Ann. Rev. Immunol. 9, 617-648.
24. Jiang, H., Robey, F. A., and Maciag, T. (1993) J. Cell Biol. 121, 603-606.
25. Smith, R. D., and Ooi, C. E., Weiss, J., Elsbach, P., and Conlon, P. J. (1992) Infect. Immun. 60, 4754-4761.
26. Weiss, J., Elsbach, P., Olson, I., and Odelberg, H. (1978) J. Biol. Chem. 253, 2659-2672.