Antibacterial 15-kDa Protein Isoforms (p15s) Are Members of a Novel Family of Leukocyte Proteins

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We have previously described the isolation and initial characterization of functionally distinct 15-kDa protein isoforms (p15s) from rabbit polymorphonuclear leukocytes (PMN) that bind with high affinity to Escherichia coli and modulate the antibacterial actions of other leukocyte proteins on this Gram-negative bacterium. We now report the cloning and sequencing of two distinct cDNAs from a rabbit bone marrow library that encode p15s differing at only 2 residues (His-3, Arg-88 versus Arg-3, Trp-88). Tryptophan-directed chemical cleavage of two isoforms purified from a single rabbit confirms the existence of multiple isoforms with distinct function and primary structure in a single rabbit. The p15 cDNAs encode putative signal peptides of bovine PMN, and cathelin, an 11-kDa cysteine protease inhibitor from porcine leukocytes, suggesting the existence of a novel family of leukocyte proteins with LPS-binding, antimicrobial, and protease-inhibitory activities.

Mobilization of polymorphonuclear leukocytes (PMN) is an important early event in the inflammatory response of the host to microorganisms and their component molecules. The granules of PMN contain an array of proteins and peptides that participate in inflammation both within the phagolysosome and extracellularly. Among the identified granule constituents of PMN are several antimicrobial proteins, including the bactericidal/permeability-increasing protein (BPI; Ref. 1), defensins (2), and azurocidin (3).

We have recently reported (4) the isolation of novel 15-kDa proteins (p15s) from rabbit PMN that are nearly identical in amino acid composition and N-terminal amino acid sequence. Despite their close similarity, the two isoforms can be separated chromatographically and can also be distinguished functionally. Although isolation of the p15s is based on their high affinity, Mg<sup>2+</sup>-reversible binding to Escherichia coli, neither protein exhibits antibacterial activity by itself under our usual assay conditions. Both proteins, however, modulate the action of BPI and azurocidin on E. coli and, in this regard, the two isoforms differ. The isoforms have opposite effects on the two successive stages of BPI action on E. coli, potentiating the early bacteriostatic and envelope-altering effects, but, at higher concentrations, inhibiting the later bactericidal action of BPI (as well as the bactericidal effects of azurocidin). Whereas one isoform strongly potentiated the early BPI effects (reducing the required dose of BPI by up to 20-fold), the other isoform had only weak potentiating activity. To further define the structural and functional characteristics of these proteins, we have determined in this study the primary structure, cellular and subcellular localization, and the lipopolysaccharide (LPS)-binding properties of p15 isoforms. Structural analysis reveals that the p15s are related to three other proteins of leukocytes from three animal species, suggesting that we have identified a novel family of leukocyte proteins.

MATERIALS AND METHODS

Purification of p15s—The 15-kDa protein isoforms were purified from rabbit peritoneal exudate PMN and their identity monitored by SDS-PAGE, reverse-phase high performance liquid chromatography, and BPI modulating assays as previously described (4).

Amino Acid Sequencing—The N-terminal residues of chromatographically separated p15s were analyzed by automated sequential Edman degradation on a Porton 290E protein peptide microsequencer.

Isolation of cDNAs—A λgt11 cDNA library prepared from the bone marrow of a New Zealand White rabbit was kindly provided by Dr. Tomas Ganz (Wilk Rogers Pulmonary Research Laboratory, UCLA) and Steve Leong (Department of Developmental Biology, Genentech). The library was initially screened by standard methods (5) with a synthetic 8-base pair (bp) oligonucleotide probe constructed on the basis of N-terminal sequencing (27 residues) of a mix of p15 isoforms. The sequence was 5'-ATT CCT CAC AGG AGG CTG TAG GAG GAG GTG GCT CAG CTC TAG TAC AAT GAA GAC CAG CCT GGC AAT CCC 3'. This probe was end-labeled with [32P]ATP using polyuridine kinase (GIBCO/BRL). A second screen of the library was performed using the N-terminal AspHII fragment of the p15-Arg.
3, Trp-88) cDNA labeled by the random hexamer method (Pharmacia LKB Biotechnology Inc.).

cDNA Sequencing—cDNAs from positively hybridizing plagues were sequenced either directly in the λ vector by the thermal cycle sequencing method as described by the manufacturer ( Gibco/BRL ) or after subcloning into M13 mp18 by the dideoxy-chain termination method (14). For all sequences shown, both strands were sequenced at least twice.

Sequence Analysis—DNA and deduced protein sequences were analyzed using the GCG Sequence Analysis Software Package (7). Protein homologies were identified using the FASTA and TFASTA programs (8) on the GenBank, Swissprot, and National Biomedical Research Foundation data bases. Sequences were aligned based on a pairwise comparison using GAP and were manually positioned using the LINEUP and PRETTY programs. The program PRETTYBOX (courtesy of Richard Westerman, AIDS Center Laboratory for Computational Biochemistry, Purdue University) was used to obtain output for multiple sequence alignments.

Tryptophanyl Cleavage Analysis of Purified Proteins—N-Chloro-

succinimide/urea (NCS/urea) was used for the selective cleavage of tryptophanyl peptide bonds (9, 10) according to Lischwe and Ochs (11) with slight modifications. After SDS-PAGE of 15-kDa isoforms (1 μg), gels were stained with 0.05% Coomassie Brilliant Blue (Bio-Rad) in 40% methanol, 10% acetic acid for 20 min at 37 °C. After destaining protein bands were cut out and stored at −20 °C until use. Gel slices were first washed with distilled water for 20 min with one change of water (2 ml was used with urea-containing buffers), then 1 ml of distilled water, 1 ml of acetic acid for 20 min with one change. Slices were then incubated for 10 min at 37 °C in urea buffer with or without 0.015 M NCS (Sigma). NCS/urea was removed by washing four times with distilled water; twice briefly and twice for 10 min. Purified proteins were eluted in 100 mM dithio-

Bromophenol blue, 3% SDS, and 0.0625 M Tris HCl, pH 6.8, for 40 min with one change. Cleavage products were resolved on 1-mm 14% polyacrylamide gels.

Binding of Proteins to LPS-coated Beads—Magnetic beads (4 × 10^7 beads, Dynal A.S., Oslo, Norway) were prepared by incubating for 48 h at room temperature on a rotator with 0.1 M sodium carbonate buffer (pH 9.5) with or without 1 mg/ml ReLPS (United States Biochemical Corp.) dispersed by sonication (Sonics Diennembrator, Fisher) at 30% maximum output for 2 min. Thereafter, the beads were collected with a magnetic particle concentrator (Dynal), washed twice with HBSS, Gihnco Laboratories, and stored at 4 °C in HBSS containing 0.1% sodium azide. Binding of the p15s (2 μg) to these beads was assessed after preincubation (37 °C, 30 min, rotation) with cell-free, protein-free clumps of a Micrococcus lysodeikticus cell wall preparation in 20 mM Tris HCl, pH 7.4, 0.001% bromophenol blue, 3% SDS, and 0.0625 M Tris HCl, pH 6.8, and 2% rhodamine-conjugated goat anti-guinea pig IgG (Kirkegaard & Perry Laboratories) in PBS, blocked with PBSA (0.2% bovine serum albumin in PBS) and 10% heat-treated normal rabbit serum and, and stained with a dilution of anti-pl5 serum or preimmune serum (0.05% in PBSA) as previously described (14). Rabbit cells were collected by sedimentation at 100 × g for 10 min from heparinized blood and from sterile inflammatory exudates 18–21 h after intraperitoneal injection of 300 ml of physiological saline containing (2.5 mg/ml). Blood cells were treated with distilled water for 30 s before reconstitution of isotonicity to reduce the amount of erythrocytes to white blood cells. Cells were resuspended in phosphate-buffered saline (PBS; 0.8% (w/v) NaCl, 0.02 KCl, 0.12 NaHPO₄, 0.02 KHPO₄, pH 7.4) to 5 × 10^7/ml. Cell smears were prepared by spinning (5 min, 500 rpm) onto microscope slides using a cytotoxicenger (Cytopsin 2, Shandon).

Release of p15s from Degranulating PMN—Rabbit PMN were collected from guinea pigs by incubation with one of the chromatographically separated species.

RESULTS

Cloning of cDNAs Encoding p15s—To define the primary structures of the p15s, we sought to isolate cDNAs encoding these proteins. A rabbit bone marrow cDNA library was screened first with a synthetic oligonucleotide probe, constructed on the basis of N-terminal amino acid sequence of purified 15-kDa proteins (4), and then with a p15 cDNA fragment. Two groups of closely similar clones were obtained, each containing cDNAs of approximately 800 base pairs. The cDNAs contain open reading frames apparently encoding p15s, as judged by both the close similarity of the deduced amino acid sequences with the N-terminal amino acid sequence(s) of purified p15s (Fig. 1). The predicted mature proteins contain 4 cysteines, no apparent N-linked glycosylation site, and an amino terminal methionine. Both groups of clones were sequencing the characteristics of a signal sequence. The encoded precursor is rich in arginine and proline and have a calculated polypeptide mass of 13.7 kDa, corresponding closely to the properties of the purified p15s (4). The two groups of clones differ only at two nucleotide positions, such that one encodes His-3 and Arg-88 and the other Arg-3 and Trp-88. Thus, p15 isoforms are very closely similar but distinct in primary structure.
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FIG. 1. Nucleotide and deduced amino acid sequences of the p15(Arg-3, Trp-88) and p15(His-3, Arg-88) cDNAs. The underlined amino acids are (nearly) identical to those of p15s as determined by amino acid sequencing. The p15(Arg-3,Trp-88) cDNA sequence is shown numbered 1-798 with the p15(His-3, Arg-88) cDNA sequence above it. Dots indicate nucleotide identity. The two divergent nucleotides in the coding region of p15(His-3, Arg-88) and the amino acids encoded are shaded above and below the p15(Arg-3, Trp-88) sequence, respectively. The most complete p15(His-3, Arg-88) clone extended from bp 15 to the poly(A) tail and was sequenced in the positive strand to bp 428 and in the negative strand from bp 67 to 98 to verify the codon representing the 3rd amino acid of the mature protein. The G nucleotides at base pairs 3, 6, and 12 indicate that the initiator ATG is in a favorable Kozak translational consensus sequence (29). The star indicates the stop translation codon and the polyadenylation signal (AATAA) is found at bp 764.

Structural Comparison of p15s Purified from a Single Rabbit—Since the bone marrow cDNA library was prepared from a single rabbit, the presence of distinct p15 cDNAs implies that a single rabbit can express multiple p15 isoforms. This was confirmed at the protein level by purification of p15s from the peritoneal exudate PMN of a single rabbit. Two distinct isoforms were recovered that correspond both chromatographically and functionally (results not shown) to the more and less active p15 species (isoforms A and B, respectively, as originally purified from pooled PMN of multiple rabbits; Ref. 4). Automated N-terminal amino acid sequencing revealed that both isoforms contained a histidine residue at position 3. However, only the "A" form of p15 was cleaved by treatment with NCS/urea (Fig. 2), generating a major fragment of ~10 kDa, consistent with the presence of a tryptophan residue at position 88 and hence showing similarity in this respect to one of the cloned forms. In contrast, the "B" form was not cleaved and may thus represent the product encoded by the p15(His-3, Arg-88) cDNA.

Cellular and Subcellular Localization—To assess the tissue distribution of p15 mRNA, a Northern blot containing total RNA from a number of organs of a New Zealand White rabbit was probed with a fragment of the p15(Arg-3, Trp-88) cDNA. Transcript(s) of 950 nucleotides were detected in bone marrow but in none of the other tissues tested: brain, lung, heart, liver, kidney, spleen, duodenum, ileum, and colon (Fig. 3). The signal was enhanced by enriching bone marrow RNA for

FIG. 2. Tryptophan-directed chemical cleavage of p15 isoforms isolated from a single rabbit. p15A (lane 1) and p15B (lane 2), were treated with NCS/urea, electrophoresed in a 14% polyacrylamide gel, and silver-stained as described under "Materials and Methods." The 15-kDa band represents uncleaved protein, while the 10-kDa band represents the major cleavage product. Detection of the smaller fragment is precluded by the dye front.
poly(A") RNA (i.e. mRNA), confirming that hybridizing RNA corresponded to mRNA (results not shown). Thus, genes encoding p15s appear to be selectively expressed by cells of bone marrow origin.

To determine which blood cells contain p15s, fixed smears of rabbit peritoneal exudate leukocytes or rabbit peripheral blood were analyzed by indirect immunofluorescence. All PMN, from peritoneal exudates (Fig. 4) as well as from peripheral blood (not shown), stained brightly, whereas red blood cells, lymphocytes and monocytes showed little or no fluorescence. Staining of PMN within a preparation and between preparations from different rabbits was similar in intensity, confined to the cytoplasm, and punctate.

The punctate cytoplasmic staining of the p15s and the presence of typical signal sequences in their cDNAs suggest that the 15-kDa proteins are granule-associated. As an additional assessment of the intracellular localization of the p15s, freshly isolated peritoneal exudate PMN were treated with cytochalasin B and fMLP to stimulate degranulation. Table I shows that extracellular release of p15s is comparable with that of β-glucuronidase and lysozyme, respectively, primary granule and both primary and secondary granule-associated proteins. Thus, the p15s appear to be granule-associated proteins of PMN.

**Binding to LPS**—The high affinity binding of the p15s to *E. coli* is the basis for their isolation and suggests that these strongly basic proteins are attracted to the negatively charged LPS in the outer membrane of the Gram-negative envelope. To more directly assess the ability of the p15s to bind to LPS, the two isoforms purified from a single rabbit were incubated with LPS-coated magnetic beads. Both isoforms bound to LPS-coated beads but not to the control beads (Fig. 5). The proteins also bind to isolated LPS in solution, since preincubation with soluble ReLPS almost completely inhibited binding of the p15s to the LPS-coated beads.

**Structural Homologies**—A computerized homology search revealed that the p15s are structurally related to three other leukocyte proteins (Fig. 6): 1) CAP-18 (30% identity) an 18-kDa protein from rabbit PMN, reported to exhibit lipopolysaccharide (LPS) binding and neutralizing activity (19); 2) pro-indolicidin (31% identity), a 16-kDa antimicrobial peptide precursor from bovine PMN (20, 21), and 3) cathelin (34% identity), an 11.7-kDa protein from porcine leukocytes with potent cysteine protease inhibitory activity toward papain and cathepsin L (22, 23). Among these homologs, CAP-18, pro-indolicidin, and cathelin are more similar to one another (51–74% amino acid identity) than to the p15s (~30% identity). Each of these proteins contain 4 cysteine residues that are aligned exactly between the p15s, CAP-18, pro-indolicidin, and, with the introduction of a 4-amino acid gap between the 2nd and 3rd cysteines, also with cathelin. Alignment of the proteins begins near their N termini either as determined by amino acid sequencing (p15s and cathelin) or by prediction of putative signal sequences and cleavage sites encoded in their cDNAs (CAP-18 and pro-indolicidin). Size differences

| Protein       | Localization | Release  |
|---------------|--------------|---------|
| LDH           | Cytosol      | 2 ± 0.3 |
| β-Glucuronidase| Primary granules | 23 ± 4 |
| Lysozyme      | Primary and secondary granules | 31 ± 1 |
| p15s          | (Granules?)  | 24 ± 8  |

**Table 1**

**Release of proteins from degranulating PMN**

The percent release of lactate dehydrogenase (LDH), β-glucuronidase, lysozyme, and p15s were measured and calculated as described under "Materials and Methods." The data represent the mean ± the standard error of the mean of four independent determinations.

**Table 2**

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| Bead Type | Ctl LPS | p15A | p15B |
|-----------|---------|------|------|
| Pre-incubation | 0 0 100 10 | 1 0 0 100 1 0 | 10 1 |

**Fig. 5. Binding of p15s to LPS.** The binding of 2 μg of p15A (lanes 1–5) or p15B (lanes 6–10) to control beads or ReLPS-coated beads was assayed after preincubation with the indicated concentrations of soluble ReLPS as described under "Materials and Methods." The sizes of molecular size markers (M) are indicated at left (in kDa).
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The cloning of two closely similar but distinct cDNAs has defined the primary structures of two p15 isoforms and two positions (His-3, Arg-88 and Arg-3, Trp-88) where they differ. Heterogeneity is also evident at the protein level in material derived from an individual rabbit, as determined by chromatographic differences and tryptophan-specific chemical cleavage of purified p15 proteins. Furthermore, an additional isoform has been identified among purified proteins from a single rabbit that contains His-3, Trp-88, suggesting allelic variability in the p15 genes and possibly, in this rabbit, expression of an allelic intermediate between the two cloned forms (Fig. 6). The very limited structural differences between the p15 isoforms, identified so far, are in marked contrast to their pronounced functional differences in potentiation of early BPI action. However, we cannot yet exclude diversity at other amino acid positions, or differences of post-translational origin that may contribute to these functional differences. The structural basis of the differences in potentiation by p15s of early BPI effects on E. coli remains to be established and must await expression and functional characterization of recombinant p15s.

The discovery of structural homology of p15s to three other PMN proteins from different mammalian species identifies a novel family of leukocyte proteins with diverse functional properties; porcine cathelin is a potent inhibitor of cysteine proteases (22, 23), a 37-residue C-terminal fragment of rabbit cathelin (24), and a tridecapeptide derived from the C terminus of bovine pro-indolicidin have been characterized of the p15s has identified a novel family of leukocyte proteins with diverse functional properties; porcine cathelin is a potent inhibitor of cysteine proteases (22, 23), a 37-residue C-terminal fragment of rabbit cathelin (24), and a tridecapeptide derived from the C terminus of bovine pro-indolicidin have been characterized. The overall structure of these proteins is highly dissimilar. Only the cationic C-terminal fragments of CAP-18 and pro-indolicidin have been isolated in pure form and tested functionally (19, 20). The N termini of these respective C-terminal fragments are only 4 and 1 residue(s) removed from the C terminus of cathelin. In view of the high degree of amino acid identity (51–74%) between these proteins from three different animal species we speculate that cathelin is in fact the N-terminal fragment of a pig homolog of CAP-18 or pro-indolicidin. If this were so, it would raise the possibility that proteins belonging to this family may be multifunctional, containing both a cysteine protease inhibitory domain and a highly cationic C-terminal region that has LPS-binding and/or antibacterial properties (Fig. 7). The strong attraction of the p15s for lipopolysaccharides is evident by their binding to isolated LPS, either immobilized on beads or in aqueous dispersion and is also apparent in the preferential binding of the p15s in crude extracts of PMN to the LPS-rich outer membrane of the E. coli envelope (4). This affinity of the p15s and CAP-18 for the negatively charged LPS is consistent with the high positive charge that is concentrated in the C-terminal ends of these two proteins (Fig. 7).

Among the biologic effects of LPS in animals is the activation of proteases in the body fluids, resulting in triggering of a complex network of proteolytic cascades that are part of the host responses to endotoxin (24, 25). In the horseshoe crab Limulus, responses to infection by Gram-negative bacteria include amoebocyte degranulation and consequent activation of a proteolytic cascade culminating in the formation of an extracellular gel (26). A Limulus amoebocyte granule-associated protein (LEBP-PI) with both LPS-binding and protease inhibitory activity has been identified raising the possibility that such bifunctional agents are important to the integration of host responses to LPS (27). In addition, a cysteine protease is required for maturation of interleukin-1β, a cytokine that is induced by LPS (28). We envision important regulatory roles for mammalian proteins that can recognize both cell-free and envelope-associated LPS and modulate the proteolytic events it initiates. In accordance with such a broad functional range is our evidence that the p15s are granule-associated proteins of PMN that are released during degranulation. This suggests that their roles can be intracellular, upon delivery to the phagosome, as well as extracellular.

Our appreciation of the complexity of the antimicrobial arsenal of the PMN continues to grow. The structural characterization of the p15s has identified a novel family of leukocyte proteins that may share more than one function.
The hypothesis that these proteins possess the structural determinants of LPS-binding, antimicrobial activity, and cysteine protease inhibition can now be further tested by investigating these functions in an experimental setting that allows direct comparison of the various members of this protein family and, once available, of their respective N- and C-terminal fragments.

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Note Added in Proof—While this manuscript was in press, it was reported that the precursors of the bovine PMN antibacterial peptides dodecapeptide (30) and Bact 31 are also members of this novel protein family.

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FIG. 7. Structural and functional characteristics of members of a novel family of leukocyte proteins. The proteins are represented by rectangular boxes with N termini on the left. The net charge of protein subregions are indicated within. In addition, the net charges of each protein within consecutive 10 amino acid stretches (e.g., 1–10, 11–20, etc.) were calculated and are represented in proportion to their absolute values (positively charged stretches, black bars above), negatively charged stretches (white bars below). Solid vertical lines, pointed to by the vertical arrow, indicate the cleavage sites of CAP-18 and pro-indolicidin.