ISOLATION OF A COMPLEMENTARY DNA CLONE ENCODING A PRECURSOR TO HUMAN EOSINOPHIL MAJOR BASIC PROTEIN

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The antimicrobial activity of polymorphonuclear leukocytes (PMN)[1] depends both on reduction products of O_2 that are generated *de novo* upon contact with the pathogen (1), and preformed polypeptides that are stored in lysosomes for delivery to nascent phagosomes (2, 3). Well-characterized microbicidal polypeptides from neutrophils include (in decreasing order of M_r): bactericidal/permeability-increasing protein (BPI, 58×10^3) (4), probably closely related to cationic antimicrobial peptide (CAP, 57×10^3 [5]); cathepsin G ($25-29 \times 10^3$) (6); lysozyme (14×10^3) (7); defensins (3.3×10^3) (8); and bactenecin ($\sim 1.6 \times 10^3$) (9). Each of these cationic proteins has a characteristic spectrum of microbicidal activity. Some selectively kill Gram-negative (BPI, CAP) or Gram-positive organisms (lysozyme); others kill both Gram-negative and Gram-positive bacteria as well as fungi (defensins, cathepsin G). Cytotoxic polypeptides from eosinophils include eosinophil-derived neurotoxin ($18 \times 10^3 M_r$) (10), eosinophil cationic protein ($17-20 \times 10^3 M_r$) (11), and major basic protein (MBP, $13 \times 10^3 M_r$) (12). The eosinophil-derived proteins reportedly lack microbicidal activity against Gram-negative or Gram-positive bacteria and fungi, although they are toxic to helminths, protozoa, and mammalian cells (13).

As part of a systematic survey of the antimicrobial proteins of human PMN (Gabay, J., R. Scott, D. Campanelli, C. Wilde, J. Griffith, M. Seeger, C. Nathan, manuscript in preparation), we purified a 14-kD protein associated with potent microbicidal activity against Gram-negative and Gram-positive bacteria and fungi. The NH_2-terminal sequence of this polypeptide proved to be identical to the NH_2 terminus of MBP (14). Additional amino acid sequencing allowed us to design oligonucleotide probes which were used to screen a cDNA library made from a promyelocytic leukemia cell line. From this library we isolated a class of cDNA clones encoding a 25-kD polypeptide, which contained in its NH_2-terminal half a sequence predicted to be hydrophilic and markedly acidic, and in its COOH-terminal half the MBP sequence, which is, in contrast, hydrophobic and very basic.

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[1] *Abbreviations used in this paper:* BPI, bactericidal/permeability-increasing protein; CAP, cationic antimicrobial protein; KU, killing units; MBP, major basic protein; PMN, polymorphonuclear leukocyte(s).

Materials and Methods

Isolation of PMN Proteins. Blood was obtained from healthy donors and processed as previously described (15). More than 98% of the cells were PMNs, of which 93–97% were neutrophils and 3–7% eosinophils. After nitrogen bomb cavitation, azurophil granules were separated on discontinuous Percoll density gradients (15). Azurophil granule extracts were prepared by freeze-thaw and sonication of the granules followed by centrifugation at 10,000 *g* for 60 min at 4°C. The pelleted material was extracted with 50 mM glycine-HCl buffer, pH 2.2 (15), centrifuged at 10,000 *g* for 20 min, and the supernatant was concentrated 20-fold using a Centricon-10 microconcentrator of 10,000 M_r cutoff (Amicon Corp., Danvers, MA).

Purification of Major Basic Protein. Approximately 1 mg of the granule membrane extract was applied to a Bio-Sil TSK-125 size exclusion column (Bio-Rad Laboratories, Richmond, CA) equilibrated in 50 mM glycine/0.1 M NaCl, pH 2.0, and eluted at a flow rate of 0.5 ml/min. Fractions were assayed for antimicrobial activity as described below. The low molecular weight peak exhibiting both antifungal and antibacterial activity was adjusted to 0.1% TFA and further purified by reverse-phase HPLC using a Vydac wide pore C4 (250 × 4 mm) column equilbrated in 0.1% TFA. Elution of the 14-kD polypeptide was performed with a 0–48% acetonitrile gradient in 0.1% TFA at a flow rate of 1 ml/min on an HPLC System, (Beckman Instruments, Inc., Fullerton, CA). The resolved peak was lyophilized after evaporation of acetonitrile by flushing with oxygen-free nitrogen.

Amino Acid Sequence Analysis of MBP. Reverse-phase purified MBP was sequenced using a pulsed liquid phase sequenator (model 477A; Applied Biosystems, Inc., Foster City, CA). Phenylthiohydantoin analysis was performed on line using a PTH Analyzer, (model 120A; Applied Biosystems, Inc.).

Assays for Antimicrobial Activity. Bactericidal activity was tested against *Escherichia coli* K12 (strain MC 4100) and *Streptococcus faecalis* (ATCC 8043) as previously reported (15). Killing assays were performed in 50 mM citrate buffer, pH 5.5 (*E. coli*), or 50 mM phosphate buffer, pH 6.0 (*S. faecalis*). Fungicidal activity was tested against *Candida albicans* (clinical isolate from Columbia Presbyterian Hospital, New York, NY). Sabouraud dextrose broth and Sabouraud agar plates (Difco Laboratories, Detroit, MI) were used to cultivate fungi. Organisms from a single colony on agar plates were inoculated into liquid medium and cultured for 24–48 h at 37°C. Aliquots of the subculture were inoculated into fresh nutrient broth and grown to 7–8 × 10^6 organisms/ml, as determined with a counting chamber. *C. albicans* (1–2 × 10^4 CFU/ml) was incubated in 10 mM phosphate buffer, pH 5.5, for 60 min at 37°C with various amounts of granule extract. Samples were then diluted 1:10 in M63 minimal medium salts (16) and spread onto agar plates. Antimicrobial activity was expressed as killing units (KU), defined as the reciprocal of the dilution of granule extract necessary to kill 10^5 bacteria/ml in 30 min at 37°C (LD_{50}) or 10^4 fungi/ml in 60 min at 37°C. Specific activity was expressed as KU/mg protein.

Tissue Culture. The human promyelocytic leukemia cell line HL60 and the acute myelogenous leukemia cell line KGla were obtained from the American Type Culture Collection (Rockville, MD). Both were maintained at 37°C in suspension culture at 0.5 to 2.0 × 10^6 cells/ml in modified DME supplemented with 10% FCS, penicillin, streptomycin, and glutamine. Cells were induced to differentiate with the addition of DMSO (Sigma Chemical Co., St. Louis, MO) by resuspending cells in fresh medium containing 1.0% DMSO at a concentration of 10^6 cells/ml. Cultures were harvested at 40 h after induction, when cell viability was >85%, and RNA was prepared immediately. The human adenocarcinoma cell line SK-HEP-1 was obtained from American Type Culture Collection, and human foreskin fibroblast cells (HFF) were obtained as a primary culture at passage 9 from Dr. Joffre Baker, University of Kansas, Lawrence, KS. These cells were carried as monolayer cultures in complete DME supplemented with 10% FCS.

Preparation of mRNA and Northern Blot Analysis. Cytoplasmic RNA was isolated from clarified extracts of cells lysed with 0.4% NP-40 (Sigma Chemical Co.) in the presence of 10 mM vanadyl complex by extraction with phenol-chloroform and precipitation with ethanol (17, 18). Polyadenylated mRNA was purified by oligo(dT) chromatography (19). Poly(A)-containing cytoplasmic RNA was denatured in formamide/formaldehyde buffer and 10-μg samples were applied to a 1.5% agarose formaldehyde gel and electrophoresed at 60 V for 15 h (20). The

RNA was electrotransferred to a Genescreen membrane according to the manufacturer's recommendation (DuPont Co., Wilmington, DE).

Construction of Human cDNA Library. cDNA was synthesized from 5 μg of mRNA using a modification of the method of Gübler and Hoffman (21). The purified cDNA product was cloned into the Eco RI site of λGT 10 according to the procedure of Huynh et al. (22). The resulting cDNA libraries contained ~2 × 10^6 independent recombinants with inserts >500 bp for the uninduced library, or inserts >300 bp for the DMSO-induced library. Approximately 10^6 recombinant phage were screened by hybridization of the 14-mer probe to duplicate filter lifts (23).

Hybridization Procedures. DNA fragment probes were hybridized under standard conditions in a buffer containing 50% formamide, 5× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 2× Denhardt's solution, 20 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.2% SDS, and 100 μg/ml yeast RNA at 42°C (18). Oligomer probe hybridization was carried out at 32°C in oligomer hybridization buffer (6× SSC, 0.2% SDS, 2× Denhardt's, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA, and 100 μg/ml yeast RNA [24]). The filters were washed at 25°C in 2× SSC, 0.1% SDS, 10 mM phosphate, and then were exposed to Kodak XAR x-ray film at −80°C. The oligomer probes were labeled by the addition of ^{32}P to the 5′ base using polynucleotide kinase and purified over a Bio-Gel P-4 spin column (18). DNA fragment probes were labeled by nick translation (25) with DNA Polymerase I in the presence of [^{32}P]dCTP and were purified over a Bio-Gel P-60 spin column.

Preparation of Human Genomic DNA and Southern Blot Analysis. High molecular weight genomic DNA was prepared by SDS-proteinase K treatment and phenol-chloroform extraction of nuclei isolated after lysis of ~10^8 cells with 0.5% NP-40 (18). The purified genomic DNA was digested to completion with the appropriate restriction enzymes according to the manufacturer's recommendation. The restricted DNA samples were extracted with phenol-chloroform, precipitated with ethanol, and dissolved in gel sample buffer. The DNA was fractionated by electrophoresis in an 0.8% agarose gel in Tris-borate buffer (18). The gel was processed and electroblotted to a Genescreen membrane as described above.

DNA Manipulations. DNA was purified from phage plate lysates of the positively hybridizing clones according to the method of Davis et al. (26) and subcloned into pUC plasmids for large-scale preparations. Plasmid DNA was purified after alkaline-SDS lysis (18) of saturated cultures of JM101, followed by polyethylene glycol precipitation. Synthetic DNA was prepared using a Gene Assembler (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) and was purified on NENSORB prep columns (DuPont, NEN Research Products, Boston, MA).

DNA Sequencing and Analysis. Restriction fragments encompassing the cDNA inserts were inserted into phage M13 vectors mp18 and mp19 (27) and sequenced by the dideoxynucleotide chain termination (28) method using *E. coli* DNA Polymerase I large fragment (DuPont Co.) or T7 polymerase (U. S. Biochemicals) according to the manufacturers' recommendations. Sequence analysis was performed using the PC Gene programs (Intelligenetics Corp., Mountain View, CA) on a Compaq 386 microcomputer.

Results

Purification of MBP. Azurophil granule membranes (derived from 4 × 10^8 PMNs) were incubated at pH 2.2 and the extracted proteins were concentrated and separated using HPLC size-exclusion chromatography, as shown in Fig. 1. Fractions were assayed for bactericidal and fungicidal activities. Both activities comigrated in two peaks, one corresponding to polypeptides of 54 kD and 25–29 kD (fractions 30–35), and a smaller peak containing species of 18 kD and 14 kD (fractions 41–43). Further analysis of the high M_r peak revealed several antimicrobial proteins which will be described separately (Gabay, J., et al., manuscript in preparation). Reverse-phase HPLC of the low M_r fraction yielded one major and two minor peaks (Fig. 2). The major component, contained in fraction 30, was lyophilized, and 90% of

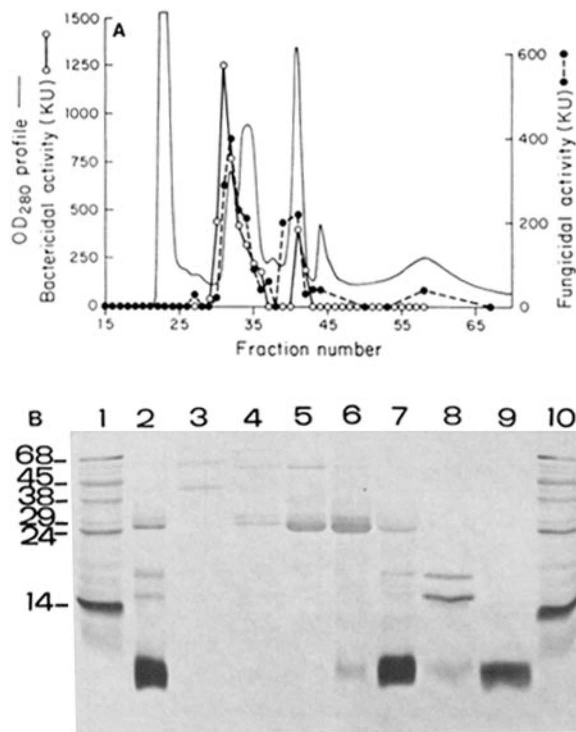

FIGURE 1. HPLC size-exclusion chromatography of azurophil membrane extract. (*A*) Profile of the OD280 and the bactericidal and fungicidal activity of the eluted fractions. (*B*) SDS-PAGE analysis of fractions eluted from the gel filtration column. Molecular weight markers (1, 10), total granule extract (2), and fractions 30,31,32,33,34,41 and 58 (3–8) were analyzed by SDS-PAGE (15% acrylamide) under reducing conditions and stained with silver nitrate.

the fraction (~300 pmol) was subjected to NH_2-terminal sequence analysis (Table I). Comparison of the NH_2-terminal sequence to known neutrophil and eosinophil proteins revealed an identity with the recently published NH_2-terminal sequence of MBP (14). SDS-PAGE analysis (Fig. 2) revealed an ~14-kD molecule, similar to the value previously reported for human MBP (29).

Antimicrobial Activity of the MBP-rich Fraction. The 14-kD, MBP-rich fraction proved to have broad spectrum antimicrobial activity against Gram-negative and Gram-positive bacteria and fungi (Table II). The LD_{50} (defined in Materials and Methods) was 1 µg/ml against *E. coli* and *C. albicans* and 0.1 µg/ml against *S. faecalis*. Since we had noted that lysozyme was often present in the MBP-rich fraction, we tested the ability of pure human lysozyme (Calbiochem-Behring Corp., San Diego, CA) to kill the same microorganisms. The LD_{50} for lysozyme was 6 µg/ml against *S. faecalis*, 0.3 µg/ml against *C. albicans*, and >1 mg/ml (no killing detected) against *E. coli*.

Oligonucleotide Probe Design. The NH_2-terminal amino acid sequence determined for MBP (Table I) contained a region (residues 32–36) suitable for the design of mixed oligomer probes. A 14-mer mixture of 16 oligomers was synthesized as the complement to the sequence encoding residues His-Asn-Phe-Asn-Ile (5' ATA/GTTA/GAAA/GTTA/GTG-3'). The 14-mer mixed oligomer probe was 5' end-labeled with $\gamma[^{32}P]$ and hybridized to a Northern blot containing *E. coli* and human rRNA, and to an array of human mRNAs isolated from HL-60, KGla, and SK hepatoma cells. Hybridization was carried out at 32°C for 24 h under standard

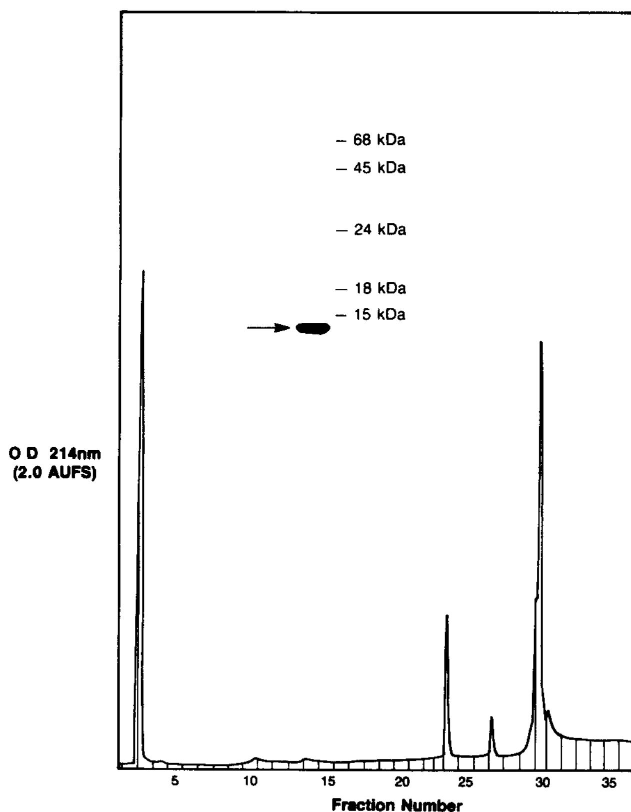

FIGURE 2. Reverse-phase HPLC purification of the 14-kD polypeptide. Fraction 41 from TSK-size exclusion chromatography was adjusted to 0.1% TFA and further purified by reverse-phase HPLC as described in Materials and Methods. Purified MBP (2 μg) was subjected to 15% SDS-PAGE (*inset*) and silver stained.

oligomer hybridization conditions (24). The oligomer probe hybridized to an ~1-kb mRNA present in HL-60 cells and very little to the control samples (data not shown). The tentative identification of a 1-kb mRNA for MBP in this preliminary analysis was unexpected, since the estimated size for an mRNA encoding a 14-kD protein is only 400–500 nucleotides.

Identification of a Full-Length cDNA Encoding MBP. Messenger RNA isolated from DMSO-induced HL60 cells was used to construct a cDNA library in λGT-10. Ap-

TABLE I

Sequence Comparison of Reverse-Phase Peak 3 to MBP

14 kD	Thr	( )	Arg	Tyr	Leu	Leu	Val	Arg	Ser	Leu
MBP*	( )	( )	Arg	Tyr	Leu	Leu	Val	Arg	Ser	Leu
14 kD	Gln	Thr	Phe	Ser	Gln	Ala	( )	Phe	Thr	( )
MBP	Gln	Thr	Phe	(Ser)	Gln	Ala	( )	Phe	Thr	( )
14 kD	Arg	Arg	( )	Tyr	Arg	Gly	Asn	Leu	Val	Ser
14 kD	Ile	His	Asn	Phe	Asn	Ile	Asn	Tyr	Arg	Ile

* From reference 14.

TABLE II

*Broad Spectrum Antimicrobial Activity of the 14-kD Polypeptide Fraction**

Pathogen	Specific activity
	KU/mg
Escherichia coli	45,000
Streptococcus faecalis	450,000
Candida albicans	45,000

* Exclusively composed of MBP (80%) and lysozyme (20%).

proximately 10^6 independent recombinant clones having a minimum insert size of 300 bp were screened for the MBP sequence by hybridization to the mixed 14-mer probe. Three cDNA clones that hybridized to the oligomer were plaque purified and found to contain inserts ranging from 400 to 550 bp. The clones were sequenced and found to contain an open reading frame that matched the NH_2-terminal sequence of MBP, beginning, in the longest clone, at residue 14 of 14-kD protein and ending at a TGA stop codon 313 bp downstream. The 3' end of these clones had an additional 110 bp of noncoding sequence, followed by a stretch of poly(A). It thus appeared that these clones represented an incomplete MBP cDNA, lacking the 5' noncoding and NH_2-terminal coding portion.

To determine the actual size of MBP mRNA, the insert fragment from the 550 bp clone was labeled by nick-translation and hybridized to a Northern blot containing induced and uninduced HL-60 mRNA. A prominent hybridizing 1-kb mRNA species could be observed, identical in size to the mRNA species that hybridized to the mixed oligomer probe. To identify a full-length MBP cDNA clone, a λGT-10 cDNA library was constructed using uninduced HL-60 mRNA and screened using the 550-bp MBP probe. 12 positive clones were plaque purified. Seven were analyzed by Southern mapping and found to contain inserts of 850–900 bp, representing candidates for full-length MBP cDNA clones. A restriction map of a representative cDNA insert is shown in Fig. 3 *A*. Four of these clones were sequenced after subcloning into M-13 vectors. The sequence of the full-length cDNA is shown in Fig. 3 *B*. An open reading frame encompassing the NH_2-terminal amino acid sequence of MBP extends from the start of the clone through nucleotide position 715. The first ATG codon, at position 49, is surrounded by sequences that closely resemble consensus mammalian translational initiation codons (GCCACCATGG) (30, 31). There is a region resembling the hexanucleotide polyadenylation signal sequence (AATAAA) (32) at position 805, which is followed 13 bp later by a stretch of adenosine residues. This suggested that the entire coding sequence was contained in the 850-bp MBP cDNA clone.

The deduced amino acid sequence beginning at the initiation codon at position 49 is followed by a 15–amino acid hydrophobic signal peptide, a 90–amino acid NH_2-terminal domain containing one potential *N*-linked glycosylation site (at nucleotide position 304), and the 117 residue COOH-terminal domain which contains the previously described MBP (14). The NH_2-terminal amino acid sequence of the 14 kDa MBP is thus contained within a 222 residue pre-pro-form, bounded by the Met initiation codon at position 49 and the translational termination codon at position 715. The pro-form of MBP is predicted to be 23 kD and the mature form

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A
BglII          HindIII    PstI  SstII
       ATG                               TGA
                                             AAAAA

B
                                                                                   
         10          20          30          40          50          60          70          80          90
          |           |           |           |           |           |           |           |           |
GGG AAG ATC TAA AGA CCC AGG AAG GTC TCT GGG TGG GAT AAA GCC AAG ATG AAA CTC CCC TTA CTT CTG GCT CTT CTA TTT GGG GCA GTT
                                                                MET Lys Leu Pro Leu Leu Leu Ala Leu Leu Phe Gly Ala Val

        100         110         120         130         140         150         160         170         180
          |           |           |           |           |           |           |           |           |
TCT GCT CTT CAT CTA AGG TCT GAG ACT TCC ACC TTT GAG ACC CCT TTG GGT GCT AAG ACG CTG CCT GAG GAT GAG GAG ACA CCA GAG CAG
Ser Ala Leu His Leu Arg Ser Glu Thr Ser Thr Phe Glu Thr Pro Leu Gly Ala Lys Thr Leu Pro Glu Asp Glu Glu Thr Pro Glu Gln

        190         200         210         220         230         240         250         260         270
          |           |           |           |           |           |           |           |           |
GAG ATG GAG GAG ACC CCT TGC AGG GAG CTG GAG GAA GAG GAG GAG TGG GGC TCT GGA AGT GAA GAT GCC TCC AAG AAA GAT GGG GCT GTT
Glu MET Glu Glu Thr Pro Cys Arg Glu Leu Glu Glu Glu Glu Glu Trp Gly Ser Gly Ser Glu Asp Ala Ser Lys Lys Asp Gly Ala Val

        280         290         300         310         320         330         340         350         360
          |           |           |           |           |           |           |           |           |
GAG TCT ATC TCA GTG CCA GAT ATG GTG GAC AAA AAC CTT ACG TGT CCT GAG GAA GAG GAC ACA GTA AAA GTG GTG GGC ATC CCT GGG TGC
Glu Ser Ile Ser Val Pro Asp MET Val Asp Lys Asn Leu Thr Cys Pro Glu Glu Glu Asp Thr Val Lys Val Val Gly Ile Pro Gly Cys

        370         380         390         400         410         420         430         440         450
          |           |           |           |           |           |           |           |           |
CAG ACC TGC CGC TAC CTC CTG GTG AGA AGT CTT CAG ACG TTT AGT CAA GCT TGG TTT ACT TGC CGG AGG TGC TAC AGG GGC AAC CTG GTT
Gln Thr Cys Arg Tyr Leu Leu Val Arg Ser Leu Gln Thr Phe Ser Gln Ala Trp Phe Thr Cys Arg Arg Cys Tyr Arg Gly Asn Leu Val

        460         470         480         490         500         510         520         530         540
          |           |           |           |           |           |           |           |           |
TCC ATC CAC AAC TTC AAT ATT AAT TAT CGA ATC CAG TGT TCT GTC AGC GCG CTC AAC CAG GGT CAA GTC TGG ATT GGA GGC AGG ATC ACA
Ser Ile His Asn Phe Asn Ile Asn Tyr Arg Ile Gln Cys Ser Val Ser Ala Leu Asn Gln Gly Gln Val Trp Ile Gly Gly Arg Ile Thr

        550         560         570         580         590         600         610         620         630
          |           |           |           |           |           |           |           |           |
GGC TCG GGT CGC TGC AGA CGC TTT CAG TGG GTT GAC GGC AGC CGC TGG AAC TTT GCG TAC TGG GCT GCT CAC CAG CCC TGG TCC CGC GGT
Gly Ser Gly Arg Cys Arg Arg Phe Gln Trp Val Asp Gly Ser Arg Trp Asn Phe Ala Tyr Trp Ala Ala His Gln Pro Trp Ser Arg Gly

        640         650         660         670         680         690         700         710         720
          |           |           |           |           |           |           |           |           |
GGT CAC TGC GTG GCC CTG TGT ACC CGA GGA GGC TAC TGG CGT CGA GCC CAC TGC CTC AGA AGA CTT CCT TTC ATC TGT TCC TAC TGA GCT
Gly His Cys Val Ala Leu Cys Thr Arg Gly Gly Tyr Trp Arg Arg Ala His Cys Leu Arg Arg Leu Pro Phe Ile Cys Ser Tyr

        730         740         750         760         770         780         790         800         810
          |           |           |           |           |           |           |           |           |
GGT CCC AGC CGA CAG TTC AGA GCT GCC CTC TCC TGG GCA TGG CCT CCC CTC CTC TGC TTG CCA TCC CTC CCT CCA CCT CCC TGC AAT AAA

        820         830         840         850
          |           |           |           |
ATG GGT TTT ACT GAA AAA AAA AAA AAA AAA AAA AAC CGG AAT TCC
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FIGURE 3. Restriction map and DNA sequence of MBPλ-9. (*A*) Restriction map. The cDNA is bounded by Eco RI sites at both ends. The restriction sites used for sequencing and the region sequenced are indicated by the arrows below the map. The open reading frame is denoted by the expanded region. The hydrophobic sequence identified by computer search as the secretory signal is shown by the shaded region. The 14-kD MBP sequence is denoted by the crosshatched region. (*B*) Nucleotide sequence and deduced amino acid sequence of MBP1-9 insert. The entire sequence was determined by the dideoxy chain termination method after subcloning restriction fragments into M13 mp18 and mp19 vectors. Numbers refer to the nucleotide position immediately after the Eco RI cloning linker. The Met initiation codon is underlined, as is the amino acid sequence of the 14-kD protein determined by Edman degradation.

14 kD. There are 12 cysteine residues contained within the pro-form, 9 of which are located in the MBP domain.

Analysis of MBP mRNA. The expression of MBP mRNA in a variety of cell lines was analyzed by Northern blot hybridization to the 850-bp cDNA clone. Poly(A)-containing cytoplasmic RNA was isolated and fractionated by electrophoresis on

denaturing formaldehyde gels. The RNA was then electroblotted to a membrane and hybridized to ^{32}P-labeled probe prepared by nick translation of the full-length MPB clone. Fig. 4 shows that MBP mRNA appears as an ~1,000-nucleotide species that was detected only in HL-60 cells. Uninduced HL-60 cultures (lane *2*) contained higher levels of this mRNA than either of the DMSO-induced HL-60 cultures (lanes *3* and *4*). Lane *2*, in addition, contained a hybridizing RNA species migrating as a broad band at 500 nucleotides. Comparable quantities of RNA from SK-Hep cells (lane *1*) or KG-1a, an acute myelogenous leukemia cell line (lane *5*), did not show detectable levels of MBP mRNA. The Northern hybridization results are in good agreement with the predicted size of the MBP mRNA of 1,000 bases, obtained by the addition of 150 bases of poly(A) to the 850-base cDNA clone.

Southern Analysis of the MBP Gene. The structure of the MBP gene was examined by restriction analysis and Southern blot hybridization. Genomic DNA samples isolated from human fibroblasts or from HL-60 cells were digested to completion with restriction endonucleases and fractionated by agarose gel electrophoresis. The DNA was electroblotted from the gel to a membrane filter and hybridized under standard conditions to the ^{32}P-labeled MBP cDNA probe. The resulting autoradiograph (Fig. 5) is consistent with the MBP gene having a relatively simple structure. The Eco RI pattern (lane *1*) consists of two bands of ~6.5 and 3.8 kb, which suggests that the gene contains at least one intron, since the cDNA clone does not have internal Eco RI sites. As predicted from the single Hind III site in the cDNA, two Hind III fragments were detected (lane *2*, human fibroblast DNA; lane *4*, HL-60 DNA) that are ~5.5 and 2.7 kb. Only one band of slightly less than 3 kb in size was found in the Bam HI digest. These results suggest that the MBP gene is <3 kb long and contains at least one intron. Although not conclusive, this analysis is consistent with MBP being a single copy gene. The restriction patterns of both HL-60 and HFF

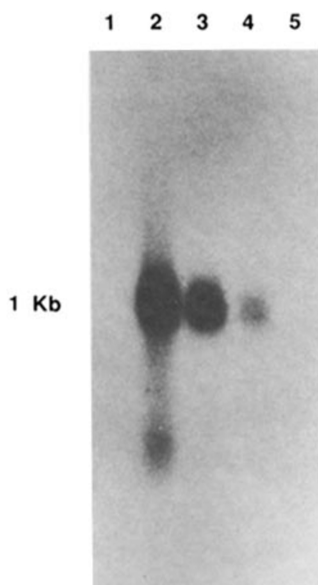

FIGURE 4. MBP Northern blot. Poly(A) cytoplasmic RNA samples, ~10 μg per lane, were fractionated on a 1.5% agarose formaldehyde gel and electroblotted to a membrane filter. The RNA blot was hybridized to nick-translated 850-bp MBP cDNA fragment under stringent conditions. The filter was washed and exposed to x-ray film. RNA size markers were visualized under UV light after staining with ethidium bromide. (Lane *1*) SK-Hep RNA, (lane *2*) uninduced HL-60 RNA, (lane *3*) DSMO-induced HL-60 RNA (72 h), (lane *4*) DMSO-induced HL-60 RNA (40 h), (lane *5*) KG1A RNA.

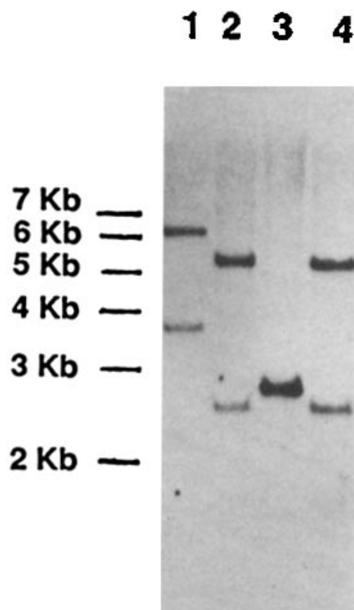

FIGURE 5. MBP Southern blot. Human genomic DNA was digested to completion with Eco RI, Hind III, or Bam HI restriction endonucleases and 15 μg of each sample was run on an 0.8% agarose gel. The DNA was electrophoretically transferred to a membrane filter and hybridized under stringent conditions to the ^{32}P-labeled 850-bp MBP probe. (Lane *1*) Eco RI digest of HFF DNA, (lane *2*) Hind III digest of HFF DNA, (lane *3*) Bam HI digest of HL-60 DNA, (lane *4*) Hind III digest of HFF DNA.

DNA are identical, demonstrating that rearrangement of the MBP gene has not likely occurred in the HL-60 cell line.

Discussion

The sequence of the NH_2-terminal 40 amino acids of the purified 14-kD protein corresponds exactly to residues Thr^{106}-Ile^{145} predicted from the cloned cDNA, and matches the recently published N-terminal sequence of human MBP (14). The predicted size of the polypeptide spanning Thr^{106}-Tyr^{222} is 13,800, similar to the apparent M_r of the 14-kD protein and to values reported for MBP (29). The predicted amino acid composition of the deduced sequence corresponds closely to that reported for human MBP (12).

The most surprising finding of this study was that human MBP is likely synthesized as an ~25-kD primary translation product and subsequently cleaved to release the mature 14-kD form. The deduced amino acid sequence of the MBP cDNA from HL-60 cells contains a putative leader sequence indicative of a secreted protein, with a predicted cleavage site for signal peptidase after amino acid 15 or 16. There is a potential *N*-linked glycosylation site at amino acid position 86, which may contribute to the solubility of pro-MBP in aqueous solutions. The predicted 23-kD pro-MBP contains 12 cysteine residues, of which 9 are in the MBP domain. The assymmetric distribution of cysteines would place at least one free cysteine in each domain (NH_2-terminal and COOH terminal) of pro-MBP. The presence of free sulfhydryls in mature MBP is consistent with its reported tendency to aggregate (33).

Based on computer analysis, the putative secreted form of the 23 kD molecule has a predicted pI of 6.0, whereas the 14-kD MBP domain has a predicted pI of 11.1. As might be expected, the NH_2-terminal domain is predicted to have an ex-

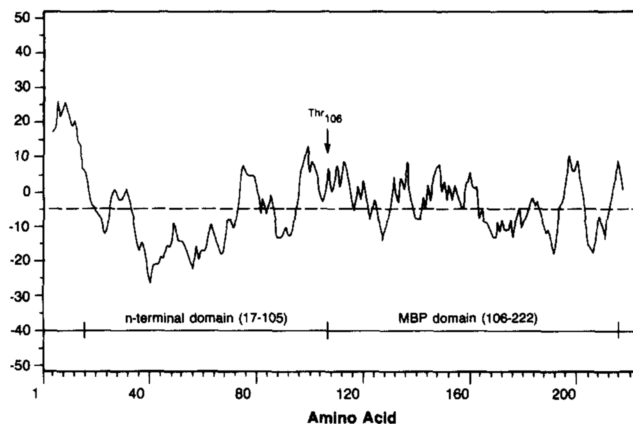

FIGURE 6. Hydropathic plot of the MBP precursor sequence. The full-length amino acid sequence encoded by the gene described in Fig. 3 was analyzed for hydrophilic/hydrophobic domains by the method of Kyte and Doolittle (50).

tremely acidic pI of 3.7. Likewise, the hydropathy plot (Fig. 6), indicates that the NH_2-terminal domain is hydrophilic, whereas the MBP domain (residues 106-222) is markedly hydrophobic. It would thus appear that the MBP is initially contained within a bipolar molecule that is likely to have markedly different biological properties from mature MBP.

The bipolar nature of pro-MBP is reminiscent of the characteristics of BPI. All the microbicidal activity of BPI toward Gram-negative bacteria is expressed by the cationic NH_2-terminal 25-kD portion of the molecule (34). The COOH terminus predicted from the cDNA for BPI is slightly negatively charged, and contains many hydrophobic residues (35). Pro-forms also exist for myeloperoxidase, another constituent of the azurophil granule (36); magainin, a 3-kD bactericidal polypeptide from the skin and mucous membranes of frogs (37); nisin (38), subtilin (38), and epidermin (39), antibiotic polypeptides of bacterial origin; and cecropin B, a bactericidal protein from the moth *Cecropia* (40). In none of these cases has a function been definitively ascribed to the pro-form. Possible functions include targeting of the antimicrobial protein to the proper subcellular compartment or to its proper position within that compartment, maintenance of the cytotoxin in a latent state to minimize autotoxicity, participation in the activation of the molecule or its transfer to the target cell, or performance of an additional function that may be unrelated to microbicidal activity.

MBP was isolated in these studies from a membrane fraction of azurophil granules purified from PMN preparations that contained 3–7% eosinophils. Since the major source of MBP has been shown to be eosinophils and basophils, it is most likely that the low level of MBP found in our neutrophil preparations was due to the copurification of eosinophil granule proteins with the azurophil granules. However, our results do not exclude the possibility that MBP is a minor constituent of neutrophils, despite the reported lack of reactivity of anti-MBP antisera with neutrophils by immunofluorescence (41) and immunoelectronmicroscopy (42). Thus, it remains unclear whether primary eosinophils and basophils are the only PMN that contain MBP (43), or whether MBP is a consituent of all three types of PMN. We are currently trying to resolve this issue by separating eosinophils and neutrophils on a preparative scale without rupturing either cell population.

Finally, it was surprising that MBP was associated with broad spectrum antimicrobial activity toward bacteria and fungi, properties MBP was previously reported to lack. Upon closer study, we discovered that our preparations often were contaminated with lysozyme. However, authentic lysozyme lacked the ability to kill Gram-negative bacteria, and was far less potent at killing Gram-positive bacteria than the 14 kD fraction. It remains for further study to define the contribution of MBP to these results and to determine whether MBP and lysozyme act synergistically. Preliminary findings with pure MBP, free of lysozyme, confirm the results reported here with the 14 kD fraction, and will be presented elsewhere as part of a more definitive analysis of MBP bioactivity.

In previous studies, MBP was toxic to schistosomula of *S. mansoni* (44), trypomastigotes of *T. cruzi* (45), and a variety of mammalian cells (46) at ~100-fold higher concentrations than required in the present studies with bacteria and fungi. Proteins that crossreact immunologically with MBP have been observed at concentrations above 10 μg/ml in sputa, pleural fluid, and sera of patients with asthma, eosinophilic pneumonia, and hypereosinophilia (47, 48). The serum level of MBP-related molecules normally reaches 7.5 μg/ml shortly before parturition (49). It will be of interest to express both recombinant MBP and recombinant pro-MBP, in order to compare their antimicrobial properties, toxicities, and mechanisms of action, and to define their relatedness to the cytotoxic polypeptides of mononuclear leukocytes.

Summary

A 14-kD protein was purified from human PMNs and its NH_2-terminal sequence was determined. Comparison of a portion of the NH_2-terminal sequence of this protein to the recently reported NH_2-terminal sequence of eosinophil major basic protein (MBP) showed them to be identical. To aid further characterization of the structural and functional properties of this molecule, we isolated from an HL-60 cDNA library a single class of cDNA clones whose sequence matched exactly the NH_2-terminal amino acid sequence of the 14-kD polypeptide. Northern analysis of HL-60 cells suggests that MBP is constitutively expressed in HL-60 cells and is highly transcribed from a single copy gene. The sequence of the full-length cDNA clones predicts that MBP is synthesized as a 23-kD precursor form (pro-MBP) which is subsequently cleaved to release the mature 14-kD MBP. The putative pro-MBP has a predicted pI of 6.0, but both the charged and the hydrophobic residues are assymetrically distributed, creating a bipolar molecule. The NH_2-terminal half has a predicted pI of 3.7 and is hydrophilic, while the COOH-terminal half (corresponding to mature MBP) has a predicted pI of 11.1 and is hydrophobic.

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Note added in proof: After this paper was submitted, the complete amino acid sequence of mature MBP and the cloning of the MBP precursor were reported (51, 52).

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