A Novel and Highly Divergent Homolog of Human Eosinophil Granule Major Basic Protein*

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Eosinophils are important effector cells in defense against helminth infection and in allergic diseases. To identify novel eosinophil proteins, large scale sequencing of a cDNA library prepared from interleukin-5-stimulated umbilical cord precursor cells was performed, and the major genes expressed by maturing eosinophils were determined. This resulted in the identification of a cDNA with 64% identity to human prepro-major basic protein (hprepro-MBP). This cDNA was designated hprepro-MBP homolog (hprepro-MBPH). Interestingly, the calculated pI values for hMBPH and hMBP differed by >100-fold, with pI values of 8.7 and 11.4, respectively. Given this pronounced basicity difference, the homolog transcript’s abundance (1.1%), and MBP’s critical role in eosinophil biological activity, we further characterized the homolog. Reverse transcription-polymerase chain reaction detected transcription of hprepro-MBP in bone marrow only, and this result was confirmed by analysis of a large cDNA data base (electronic Northern). hMBPH was isolated from human eosinophil granule lysates, and its identity was verified by amino acid sequencing and by mass spectrometry. Analyses of the biological activities showed that hMBPH had effects similar to hMBP in cell killing and neutrophil (superoxide anion production and interleukin-8 release) and basophil (histamine and leukotriene C4 release) stimulation assays, but usually with reduced potency. Overall, this novel homolog’s unique physical properties indicated that the high net positive charge of hMBP is important but not essential for biological activity.

The eosinophil functions as a major effector cell in pathologic states (1, 2) including those associated with allergy and with pronounced eosinophilia and as a protective cell against helminthic infection (1). The major basic protein (MBP)1 is an abundant granule protein and an important mediator of eosinophil biological function (1–3). It constitutes the crystalloid core of the eosinophil-specific granule and is the principal protein composing the granule (3, 4). Human MBP (hMBP) is a 13.8-kDa protein with a calculated isoelectric point near pH 11. The cDNA sequence predicts initial translation of a 25.2-kDa prepro-form containing an additional signal sequence (“pre”) and a highly acidic (“pro”) domain. MBP possesses numerous cytotoxic properties (3) including the ability to damage helminths, bacteria, protozoa, and mammalian cells, and it is implicated as a mediator of pathology. For example, MBP is deposited on damaged lung epithelium of patients with asthma, in the upper dermis of the skin in patients with atopic dermatitis, and in cardiac tissues of patients with eosinophilic endomyocardial disease (5–7). Furthermore, MBP stimulates bronchial hyperreactivity similar to that observed in asthma (3, 8, 9). MBP also activates cells and stimulates cytokine production by them. Examples include the regulation of cytokine production from neutrophils,2 eosinophils (11), and lung fibroblasts (12) and stimulation of biological mediator release from basophils and mast cells (13). Additionally, blood concentrations of the pro-form of MBP (pro-MBP) increase dramatically during human pregnancy (14, 15), and this results from a striking expression of pro-MBP by the placental X cell (16, 17). Altogether, these observations support MBP’s involvement in the pathophysiology of allergic and eosinophil-associated diseases and its beneficial role in resistance to parasitic disease.

To further characterize gene products important in eosinophil function, we sought to identify novel genes transcribed by precursor blood cells cultured in the presence of interleukin (IL)-5, a cytokine that causes eosinophil proliferation, differentiation, and activation (3). The approach chosen involved large scale cDNA sequencing (18) of thousands of plasmid-inserted cDNAs generated from the IL-5-differentiated precursor cells. The resulting frequency profile of the sequenced cDNAs, referred to as a Transcript Image, provides a quantitative and qualitative record of gene expression (18, 19). Analysis of the Transcript Image of the IL-5-differentiated precursor cells revealed a transcript with significant homology to human prepro-major basic protein (hprepro-MBP). This transcript was named hprepro-MBP homolog (hprepro-MBPH). Compared with previous MBP molecules, the deduced amino acid sequence corresponding to the MBP section of the hprepro-MBPH transcript showed a marked reduction in the number of positively charged amino acids. This marked basicity difference, the transcript’s high abundance, hMBP’s association with various health and...
Human Major Basic Protein Homolog

Experimental Procedures
IL-5-induced Differentiation of Umbilical Cord Progenitor Cells—Differentiating eosinophils were produced in vitro by stimulation of umbilical cord progenitor cells (UCC) with recombinant human IL-5, a generous gift from Schering-Plough Research Institute, Kenilworth, N.J., essentially as described previously [20–22]. Eosinophil differentiation was monitored with cyanide-resistant peroxidase staining [22], a marker for eosinophil peroxidase. By days 10–12, 60–100% of the cultured cells stained positive for eosinophil peroxidase. Cells from the recombinant human IL-5-stimulated cultures were pelleted and lysed in 4× granulocytic thiocyanate at 10⁷ cells/ml. Lysates were stored at −70 °C until processing. Total RNA was extracted using standard TrCl methods [24]. One hundred micrograms of pooled total RNA was utilized for construction of a cDNA library for Transcript Image generation.

Transcript Image Generation—Total RNA, prepared from recombinant human IL-5-stimulated UCC populations following guanidinium lysis as described above, was subjected to cDNA treatment, and poly-A tails were isolated using Qiagen Oligotex (Qiagen, Inc., Chatsworth, CA). Polyadenylated RNA was then converted to cDNA using the SuperScript Plasmid System (Life Technologies, Inc.), where the plasmid cloning vector was a polynucleotide-modified pSPORT-pIII. Prior to cloning, cDNAs were size-fractionated on Sepharose CL-4B (Amersham Pharmacia Biotech).

Following transformation, individual colonies were picked, and plasmid DNA was released and purified using the REAL Prep 96 plasmid kit (Qiagen). Sequencing of cDNA using ABI 377 sequencers and standard ABI sequencing protocols was performed on 15,000 plasmid DNAs. All plasmid sequences were screened to identify putative cDNA inserts followed by iterative screening of these cDNAs against public nucleic acid and protein databases for sequence identification. This process yielded a total of 8,146 useable sequences (i.e., nonmitochondrial and nonribosomal sequences). The number of times a unique cDNA was identified was recorded, resulting in a steady state transcription profile or image, and referred to as a Transcript Image.

mRNA Expression in Tissues—Polymerase chain reaction (PCR) primers specific for the “pro” section of hprepro-MBP or hprepro-MBP were designed using Oligo software (National Biosciences, Inc., Plymouth, MN). hprepro-MBP primer pair 5′-TCTGGAACCTCCACCTTGAGACC-3′ and 5′-GGTGTCTGGCAGATCTGGCACC-3′ and hprepro-MBP primer pair 5′-GAAGATGTGCCCCCTACCTGTG-3′ and 5′-CAGTGAAATCCTGTGACCATGC-3′ were expected to produce PCR products of 198 base pairs and 214 base pairs in length, respectively. Template cDNAs from human tissues (CLONTECH, Palo Alto, CA) were tested for the presence of hprepro-MBP or hprepro-MBP sequence: brain, lung, colon, heart, kidney, lymph node, liver, lung, ovary, peripheral blood leukocytes, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, testis, thymus, and tonsil. The Expand PCR System (Roche Molecular Biochemicals) was used with cycling parameters of initial denaturation at 94 °C for 5 min, 10 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 60 s followed by 28 cycles of 94 °C for 15 s, 75 °C for 30 s, and 72 °C for 60 s with an additional 20 s per cycle and a final extension time of 7 min at 72 °C. PCR products were visualized with ethidium bromide in 2% agarose gels.

Electronic Northern—The full-length cDNA sequence of hprepro-MBP was used to search for matching sequences (>95% identity) in Incyte’s assembled data base. This data base was composed of Transcript Image data derived from multiple tissue samples divided into 20 distinct tissue categories (see Table III). The total number of sequence matches (i.e., “absolute abundance”) and the distribution of matches across the distinct tissue samples composing a given tissue category (i.e., “found in”) were recorded.

Monoclonal Antibody Production—A peptide corresponding to a segment of the N-terminal sequence of hMBP (below; Fig. 1) with minimal homology to hMBP was synthesized and covalently coupled to ImmunoChem Research short keyhole limpet hemocyanin peptide (approximately 50 μg) in RIBI adjuvant (RIBI ImmunoChem Research, Hamilton, MT) was injected intraperitoneally into five BALB/c mice (Charles River Laboratories, Wilmington, MA) monthly for 3 months followed by a final intravenous injection of approximately 50 μg of keyhole limpet hemocyanin peptide in saline 3 days prior to harvesting the spleens (25). Spleen cells were fused to FO myeloma cells and cultured as described previously (25, 26). Conditioned media were screened for reactivity to the immunizing peptide using the F.A.S.T. system (Falcon Assay Screening Test; Becton Dickinson, Oxnard, CA) with the immunizing peptide covalently coupled to 125I-labeled Immune Complex Separation (MMP) (Millipore Corp., Bedford, MA). Ultimately, one hybridoma, J191–12H11, was cultured, and the antibody was purified using a POROS 20 G- Protein G column (PerSeptive Biosystems, Framingham, MA).

Purification of hMBP—Eosinophils (about 1 × 10¹¹ cells/patient, final purity of 90%) were collected by cytopheresis of patients with the hypereosinophilic syndrome (28). Cells were further processed to isolate eosinophil granules and granule proteins, as described earlier (29–31). Fractions (1 ml each) from the Sephadex G-50 column were probed for hMBP and hMBP with monoclonal antibodies J191–12H11 and J6–8A4, respectively, by Western blotting essentially as described previously (26). Fractions containing hMBP were pooled and refractionated on Sephadex G-50. Fractions positive for hMBP with J91–12H11 and negative for hMBP by Western blotting using a similar band by Coomassie Brilliant Blue R-250 (Bio-Rad) staining were pooled, concentrated, and stored at −20 °C as purified hMBP.

N-terminal Acidic Sequence—The N-terminal amino acid sequences of hMBPH and hMBP were analyzed by Edman degradation microsequencing with the PE/ Applied Biosystems 492 Procise Protein Sequencing System (Perkin-Elmer Corp./Applied Biosystems Division) using pulsed liquid chemistry in the Mayo Protein Core Facility. Data were collected and analyzed with PE/Applied Biosystems model 610 Sequencing Software. The desalted protein samples were absorbed to polyvinylidene difluoride membranes with the PE/Applied Biosystems ProSorb cartridges before sequencing.

Mass Spectrometry—A MAGIC 2000 HPLC system (Microm Biore- sources Inc., Auburn, CA) fitted with a C9 column (50 × 0.5 mm; Microm Bioreources Inc.) in series with a Finnigan MAT 900 double sector mass spectrometer (Bremen, Germany) fitted with a position- and time-resolved ion counter array detector was used to analyze purified hMBPH and hMBP. A 15-min gradient from 5 to 60% mobile phase B (89.5% acetonitrile, 7% water, 3% n-propanol, 0.5% acetic acid, 0.02% trifluoroacetic acid) in mobile phase A (2% acetonitrile, 97.5% water, 0.01% acetic acid) at a flow rate of 500 μl/min was utilized, giving an estimated gradient delay of 2 min. Effluent from the HPLC column was interfaced with the MS via a fused silica capillary (180-μm outer diameter × 25 μm inner diameter; Polymicro Technologies, Phoenix, AZ) passed coaxially through the inner bore of the electrospray needle. This needle was biased at 4.8 kV with respect to the accelerating voltage of 5 kV. Ionization was by positive ion electrospray, using a sheath liquid flow to assist nebulization (1.5 liters/min).

The mass spectrometer was operated over a mass range of 500–2500 Da at a scan rate of 5 s/decade. The position- and time-resolved ion counter detector was operated at a microchannel voltage of 820 V and a mass window of 8%.

For alkylation of protein prior to mass spectrometric analysis, hMBPH and hMBP were incubated with iodoacetamide (1A; 20 μl of IA/mole of protein) in 100 mM Tris, pH 8.0, at 37 °C for 30 min. Similarly, reduction and alkylation were performed with dithiothreitol (20 mol/ mol of protein) in 100 mM Tris, pH 8.0 at 37 °C for 30 min followed by the addition of IA (40 mol/mole of protein) and continued incubation for 30 min.

Restriction Enzyme Determination—Purified hMBPH or hMBP in acetate buffer (25 mM sodium acetate, 150 mM NaCl, pH 4.2) with an absorbance at 280 nm of about 0.75 was centrifuged at 35,000 × g for 20 min just prior to the addition of the supernatant to a 1 × 1 cm quartz cuvette. An absorbance spectrum (250–400 nm) was then acquired on a dual beam Varian CARY 2200 spectrophotometer with acetate buffer as the blank. Three 50-μl samples were taken directly from the cuvette using three separate calibrated pipetors. A portion of each sample was then subjected to amino acid analysis in triplicate with postcolumn ninhydrin detection to quantitate the total mass of protein present. Briefly, the proteins were vapor-hydrolyzed with 6 M hydrochloric acid and 1% phenol for 24 h under vacuum at 110 °C. The hydrolysates were analyzed on a Beckman 6300 amino acid analyzer (Beckman Instruments) modified with the Pickering Biosystems column, Nα-buffers (pH 3.28, 4.25, 6.40), and Trione ninhydrin reagent (Pickering Labora-
torsies, Mountain View, CA). Norleucine was the internal standard, and a sample of standardized bovine serum albumin (Pierce) was the hydrolysis control. Extinction coefficients for hMBPH and hMBP were subsequently calculated from their respective absorbance values at 280 nm corrected for light scattering (32) (correction resulted in a 3–4% reduction in the uncorrected absorbance values at 280 nm) and their average protein concentrations were determined from the three 50-μl samples. The S.D. of the average protein concentrations for hMBPH and hMBP were used to estimate the errors of their previously calculated extinction coefficients.

Theoretical molar extinction coefficients (ε) for hMBPH and hMBP were calculated using the equation ε(M) = 5540(1480[n Trp] + 1341[n Tyr] + 1531[n His]), where n Trp, n Tyr, and n His are the numbers of tryptophans, tyrosines, and disulfide linkages within the protein of interest (hMBPH: 7 Trp, 5 Tyr, and 2 S-S; hMBP: 7 Trp, 6 Tyr, and 2 S-S).

Inhibition of Incorporation of [14C]Leucine—Cytotoxic effects of hMBPH and hMBP were tested using human chronic myelogenous leukemia K562 cells (CCL-243; American Type Culture Collection, Rockville, MD) essentially as described earlier. Twenty-five microliters of these leucine-starved K562 cells (2.5 × 104 cells) were added in triplicate to a Costar half-area, flat bottom 96-well plate (Costar Corp., Cambridge, MA) followed by the addition of 2.5 μl of buffer or protein solution. The plate was incubated at 37 °C and 5% CO2 for 2 h, and then 0.1 μl of 1% sodium deoxycholate was added. After a final incubation at 37 °C for 2 h, 100 μl of 0.1% SDS was added to each well; the contents were mixed; and the solution was transferred to a 1.5-ml microcentrifuge tube. A wash of 100 μl of 1% SDS was added to each well, mixed, and transferred to its respective tube containing the first wash. Less than 1% of the radioactive counts remained in the well. Bovine serum albumin (100 μl of a 0.1% solution) was added to each tube followed by 300 μl of 20% trichloroacetic acid to precipitate protein (34). The tubes were incubated at 4 °C for 30 min and then centrifuged at 12,000 × g for 20 min. The resulting pellet was dissolved in 100 μl of 1 n NaOH, 1 ml of Ultima Gold scintillation fluid (Packard Instrument Co., Downers Grove, IL) was added, and the samples were counted for β-radiation.

Inhibition of [14C]leucine incorporation was calculated using the following equation: % inhibition of incorporation = (1 – incorporation with protein/incorporation with buffer only) × 100.

Neutrophil Superoxide Anion Production—Neutrophils (2 × 10⁶), isolated from venous blood of healthy adult donors as described previously (35), were incubated with the indicated concentrations of hMBPH or hMBP or with equivalent volumes of the sodium acetate buffer, in HEPES-albumin-glucose buffer, pH 7.4, containing 0.6 mM CaCl2 and 1 mM MgCl2 (HAGCM buffer) and 80 μM ferritoxochrome C (10 μg/ml) at 37 °C in an oscillating water bath. Total reaction volume was 0.2 ml. The reactions were stopped by centrifugation at 400 × g for 10 min at 4 °C, and superoxide anion (O2·−) release was measured as nmol of ferritoxochrome C reduced/10⁶ neutrophils/30 min as described previously (35). Spontaneous superoxide anion production was from cells incubated without stimulus.

Neutrophil IL-8 Production—Neutrophils (10⁶), isolated as described above under sterile conditions, were incubated in 24-well tissue culture plates (Costar, Cambridge, MA) with the indicated concentrations of hMBPH or hMBP, with equivalent volumes of the sodium acetate buffer or 100 nm lipopolysaccharide (Escherichia coli 055:B5) (Sigma) in RPMI 1640 containing 100 μg/ml each of penicillin and streptomycin, 2 mm L-glutamine (Life Technologies), and 10% heat-inactivated autologous serum for 2 h at 37 °C in a 5% CO2 atmosphere as described earlier. Total culture volume was 0.5 ml. Culture supernatants were collected by centrifugation at 400 × g for 10 min at 4 °C and were stored at −20 °C until measurement of IL-8 content using a specific enzyme-linked immunosorbent assay (BIOSOURCE International, Camarillo, CA). Supernatant IL-8 production was from cells incubated without stimulus.

Basophil Histamine and Leukotriene C4 Release—Basophil-containing mononuclear cell fractions were isolated from venous blood of healthy adult donors as described previously (36). Basophils were enriched by negative selection using magnetic beads (Basophil Isolation Kit; Millenyi Biotec, Auburn, CA) and the midMACS magnetic cell separation system (Miltenyi Biotec, Auburn, CA) as described previously (35), and the manufacturer. The percentage of basophils in the three experiments ranged from 59 to 88% by Alcian blue staining. Basophils (7 × 10⁴) were incubated with the indicated concentrations of hMBP or hMBPH or with volumes of vehicle buffer (25 mm sodium acetate, 0.15 μm NaCl, pH 4.2) equal to those required for protein addition in HAGCM buffer for 30 min at 37 °C in an oscillating water bath. Total incubation volume was 0.2 ml. The reactions were stopped by the addition of 0.3 ml of cold reduced HEPES-albumin-glucose buffer, pH 7.4, and centrifugation at 1600 × g for 2 min at room temperature. The leukotriene C4 (LTC4) content in an aliquot of the cell-free supernatant was measured using an enzyme immunoassay kit for LTC4/leukotriene D4/leukotriene E4 (Amersham Pharmacia Biotech) as described previously (36), and results are expressed as ng of immunoreactive LTC4/10⁶ basophils. At the basophil concentration used in this study, the LTC4, released by the baseline activated basophils, was not metabolized to leukotriene D4 or leukotriene E4 (37). Histamine content in the remainder of the cell-free supernatant was determined fluorometrically, and histamine release was calculated as the percentage of total histamine content as described previously (36). Spontaneous histamine and LTC4 release was determined with cells incubated in the absence of stimulus.

RESULTS

Identification of Human prepro-MBP Homolog cDNA—Table I shows the 23 most abundant transcripts present in IL-5-differentiated UCC as determined by Transcript Image methodology. Three of the four most abundant transcripts were essentially identical to eosinophil-specific mRNA sequences, namely hprepro-MBP, Charcot-Leyden crystal protein, and eosinophil-derived neurotoxin (EDN). Another eosinophil-specific sequence, eosinophil peroxidase, was the eighth most abundant transcript. The Transcript Image also showed the presence of a novel sequence with 49% homology at the protein level to hprepro-MBP (Table I). This sequence was designated hprepro-MBP. The hprepro-MBP and hprepro-MBP cDNA sequences were the first and fifth most abundant cDNA inserts of the 1,46 clones screened and comprised 8 and 1.1% of these cDNA inserts, respectively (Table I). Fig. 1 shows the cDNA and deduced amino acid sequence of hprepro-MBP. The coding region of the nucleotide sequence for hprepro-MBP is 64% homologous to that of hprepro-MBP when optimally aligned using the MacVector 4.5 program (Eastman Kodak Co.). Consistent with the cDNA structure of hprepro-MBP, the homolog cDNA codes for an N-terminal signal (i.e. pre) sequence, a highly acidic “pro” section, and a basic “MBP” section (Fig. 1 and Table II). In contrast, due to the diminished basicity of its MBP section, the isolectric points of hprepro-MBP and hMBPH are approximately 100-fold lower than those of hpro-MBP and hMBP, respectively (Table II).

Tissue mRNA Expression of hprepro-MBP and hprepro-MBP—From the cDNA sequences for hprepro-MBP and hprepro-MBP, PCR primers specific for each probe section were designed (see “Experimental Procedures” and Fig. 1). Fig. 2A shows the specificity of the primer pairs. First-strand cDNAs derived from various tissue types, normalized on the basis of housekeeping genes, were used as templates in PCRs (see “Experimental Procedures”), PCR products were subsequently analyzed electrophoretically. Fig. 2, B and C, show representative results for a portion of the tissues tested for mRNA expression. Amplification of a DNA fragment of the expected length (195 nucleotides for hprepro-MBP and 214 nucleotides for hprepro-MBP) at a cycle number of 38 was observed from bone marrow and placenta (Fig. 2B) and bone marrow only (Fig. 2C) for hprepro-MBP and hprepro-MBP, respectively. The tissue expression pattern for hprepro-MBP is consistent with previous reports (17, 39). Notably, no expression was detected in peripheral blood leukocytes, as expected, because of their terminal differentiation (40).

As a second assessment of the hprepro-MBP tissue expression profile, an electronic Northern was performed using the homolog sequence as the query sequence (see “Experimental Procedures” and Table III). Consistent with the PCR data of

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Human Major Basic Protein Homolog

23 most abundant transcripts of IL-5-differentiated UCC

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a All sequences listed are of human origin.
b Absolute and percentage abundance among 8,146 total clones sequenced.
c Closest matched known sequence to hpro-MBP sequence.

d Fig. 2, expression of the homolog was only detected in the IL-5-differentiated UCC transcript image data set within the hemic and immune system tissue category. This analysis included an absence of matches with Transcript Image data from multiple placenta samples contained in the “embryonic structures” tissue category.

Purification and Identification of hMBPH from Eosinophil Granules—Using the deduced amino acid sequence of hpro-MBP, an antibody (J191–12H11) specific for an N-terminal sequence of hMBPH (see above; Fig. 1) was isolated and used to probe gel filtration column fractions for hMBP (Fig. 3). Fig. 3A shows a typical elution profile of an eosinophil granule preparation fractionated over a Sephadex G-50 column (29, 31). Fractions 71–90 reacted with J191–12H11 by Western blotting (data not shown) and were pooled and fractionated over a second Sephadex G-50 column (Fig. 3B). Samples from even numbered fractions 54–80 from this second gel filtration column were electrophoresed on three SDS-polyacrylamide gels. A Western blot of one gel was tested for hMBP (Fig. 3C). Samples from even numbered fractions 54–80 from this second gel filtration column were electrophoresed on three SDS-polyacrylamide gels. A Western blot of one gel was tested for hMBP (Fig. 3C), and another was tested for hMBPH (Fig. 3D). The third gel was stained with Coomassie Brilliant Blue R-250 (Fig. 3E). Note the apparent shift from the slightly higher molecular weight bands of hMBP (fractions 54–64) to the lower molecular weight bands of hMBPH (fractions 64–80) in Fig. 3E consistent with their respective calculated molecular weights (Table IV) and the position of the trough (fractions 63 and 64) separating the two elution peaks in Fig. 3B. Fraction 64 in Fig. 3E best shows the presence of two bands. Similar elution profiles have been obtained when using eosinophil granule preparations from the four hyperergosinophilic syndrome patients tested.

To verify the identities of the proteins composing the two peaks from the second Sephadex G-50 column (Fig. 3B), fraction 57 from the ascending side of peak 1 and fraction 76 from the descending side of peak 2 were subjected to N-terminal amino acid sequencing. As shown in Fig. 4, the resulting amino acid sequences corresponded to those anticipated for the N termini of hMBP (41–43) and hMBPH. Similarly, the molecular masses determined by mass spectrometry for proteins from the ascending side of peak 1 and from the descending side of peak 2 were in general agreement with the molecular masses calculated from the primary sequences for hMBP and hMBPH, respectively (Table IV). In both cases, however, the measured molecular mass was approximately 5 Da lower than predicted. The presence of two disulfide bonds in hMBP had previously been reported (44). Thus, disulfide linkages were suspected as the cause for the reduced experimental molecular masses. Each such disulfide linkage results in the loss of two hydrogen atoms with a concurrent reduction in molecular mass of 2 Da; however, the mass accuracy obtained was insufficient to determine if two or three such linkages were present. Therefore, two further experiments were performed for each protein. Initially, the proteins were alkylated with IA and then examined by mass spectrometry (Table IV). This provided the number of reactive cysteines present within each protein (Table IV). As expected, nonreduced hMBP contained five reactive cysteines, whereas hMBPH contained six reactive cysteines. To provide the number of disulfide bonds within each protein, fresh protein samples were reduced with dithiothreitol and alkylated with IA (Table IV). These mass spectrometry data confirmed the presence of two disulfide linkages in hMBP and suggested an identical number in hMBPH.

Extinction Coefficients of Human Major Basic Proteins—To allow accurate concentration estimates of protein used in biological assays, the extinction coefficients of hMBP and hMBPH were determined (see “Experimental Procedures”). Experimental determinations yielded extinction coefficients at 280 nm of 3.67 ± 0.09 and 3.32 ± 0.04 (mg/ml)−1 cm−1 for hMBP and hMBPH, respectively. While these values are high compared with other proteins (45), they are in good agreement with theoretically calculated extinction coefficients of 3.47 and 3.46 (mg/ml)−1 cm−1 for hMBP and hMBPH, respectively, based on tryptophan, tyrosine, and disulfide linkage content.
production is a sequence of the peptide used in the J191–12H11 monoclonal antibody.

Pro-MBPH-specific PCR primers are shown using water (Water), a plasmid containing hprepro-MBP cDNA (proMBP), and a plasmid containing hprepro-MBP cDNA (proMBPH) as template in PCRs as described under “Experimental Procedures.” The positions of DNA markers (Markers) are indicated by the lines on the left side of A, from top to bottom: 310, 271/281, 234, 194, 118, and 72 nucleotides in length. hprepro-MBP primers (B) and hprepro-MBPH primers (C) were used with the first-strand cDNA from the various tissues indicated (bone marrow (BM), lymph node (LN), peripheral blood leukocytes (PBL), pancreas (PanC), placenta (Plac), prostate (Pros), skeletal muscle (SM), small intestine (SI)) in PCRs as described under “Experimental Procedures.” A list of all tissues tested for hprepro-MBP and hprepro-MBPH is provided under “Experimental Procedures.” C also shows a negative (Water) and positive (proMBPH) control as in A.

A calculated pI near 10.9 (3). Interestingly, these highly basic proteins, ECP and eosinophil peroxidase, also inhibited protein synthesis. ECP cytotoxicity was pronounced even at 1 μM, but unexpectedly plateaued at approximately 50% inhibition. The cytotoxicity of ECP was eventually lost at a concentration of 0.1 μM (data not shown). Only EDN, which has a similar mass and nearly identical calculated pI and net positive charge (+7) to that of hMBPH (Table I), showed minimal inhibition of protein synthesis. This result suggests that a positive charge per mass unit ratio similar to that of hMBPH is not sufficient for cytotoxicity. This interpretation, however, is obscured by the lack of precise characterization of the charge contribution resulting from EDN glycosylation (46, 47).

Human MBP also exhibits a variety of cytostimulatory activities (3). Fig. 6 compares the ability of hMBP and hMBPH to stimulate those cellular responses. Human MBP and hMBPH stimulated neutrophil superoxide production virtually identically (Fig. 6A). In contrast, hMBPH stimulated neutrophil IL-8 production (Fig. 6B) about half as potently as hMBP; but still comparable with that of lipopolysaccharide. Likewise, concentrations of hMBPH approximately 4- and 10-fold greater than that of hMBP were required to stimulate comparable histamine (Fig. 6C) and leukotriene C4 (LTC4; Fig. 6D) release from basophils, respectively. Each of these appears to involve an active cellular process and is not a result of cytolsis (11, 13, 35, 48). However, it appears that hMBP is inhibitory to de novo LTC4 production when present at 4 μM (Fig. 6D).

**DISCUSSION**

Previously our laboratory has characterized the principal proteins contained within the human eosinophil granule (1, 2). To search for novel eosinophil proteins we stimulated UCC with IL-5 and sequenced 15,000 plasmid cDNA inserts. The...
result of this analysis, referred to as a Transcript Image, revealed a second prepro-MBP-like gene expressed by the human IL-5-differentiated UCC. Knowledge of the cDNA sequence for this hprepro-MBP homolog allowed analysis of its tissue expression profile; its expression was detected in bone marrow only. The cDNA sequence also allowed production of a monoclonal antibody to the corresponding protein homolog in eosinophil granules. Purification of hMBPH protein permitted comparison of its activity with that of hMBP in a series of cytotoxicity and cytostimulatory assays. These data showed qualitatively similar activities between hMBPH and hMBP but with diminished potency for the homolog in the majority of assays. Thus, the Transcript Image approach to quantitative and qualitative assessment of gene transcript expression successfully identified and led to the isolation of a novel eosinophil granule protein.

Transcript Imaging—The sequencing of approximately 10,000 clones, as in current Transcript Imaging, permits random identification of many known and novel rare messages, with a cumulative identification of rare messages for each additional sample studied. Furthermore, this procedure can detect either increased or decreased levels of gene expression that can later be compared with or reanalyzed in light of subsequent sequence data. The relatively long sequences from this electronic Northern analysis. Thus, a greater number of sequences were compared as potential matches to the homolog sequence in this tissue category.

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\text{Absolute abundance} = \frac{\text{number of distinct tissue samples that contains one or more sequences matching that of hprepro-MBP per total number of tissue samples}}{\text{number of sequences matching that of hprepro-MBP homolog allowed analysis of its tissue expression profile; its expression was detected in bone marrow only. The cDNA sequence also allowed production of a monoclonal antibody to the corresponding protein homolog in eosinophil granules. Purification of hMBPH protein permitted comparison of its activity with that of hMBP in a series of cytotoxicity and cytostimulatory assays. These data showed qualitatively similar activities between hMBPH and hMBP but with diminished potency for the homolog in the majority of assays. Thus, the Transcript Image approach to quantitative and qualitative assessment of gene transcript expression successfully identified and led to the isolation of a novel eosinophil granule protein.}}
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FIG. 4. N-terminal amino acid sequencing of hMBP and hMBPH. A portion of fraction number 57 (representing peak 1) and 76 (representing peak 2) from the second Sephadex G-50 column (Fig. 3B) were analyzed by Edman degradation microsequencing. The resulting N-terminal amino acid sequences are shown.

FIG. 5. Inhibition of cellular protein synthesis by eosinophil granule proteins. Leucine-starved K562 cells growing in log phase were exposed to buffer alone and 1 μM (open bars), 3.3 μM (hatched bars), and 10 μM (closed bars) of the test agent listed on the abscissa for 2 h followed by the addition of 0.1 μM [14C]leucine. Cyclohex., cycloheximide. After a final incubation of 2 h, total protein incorporation of [14C]leucine was assessed and compared with that in the presence of buffer only. The percentage of inhibition of [14C]leucine incorporation was calculated as described under “Experimental Procedures.” Each bar represents the mean percentage of inhibition calculated from two separate experiments, where each test agent was tested in triplicate at each of the three concentrations tested. S.D. values are shown by the error bars or are indistinguishable from the top of the data bars.

FIG. 6. Comparison of hMBP and hMBPH cytostimulatory effects. A, neutrophils from four donors were incubated with the indicated concentrations of hMBP (closed squares) or hMBPH (closed circles) for 30 min at 37 °C, and superoxide anion production was quantified as described under “Experimental Procedures.” Results are the mean ± S.D. after subtraction of spontaneous production (1.4 ± 1.7 nmol/10⁶ cells/30 min). B, neutrophils were incubated with sodium acetate vehicle buffer (NaOAc), hMBP (2 μM), hMBPH (2 μM), or lipopolysaccharide (LPS, 100 ng/ml) for 20 h at 37 °C, and IL-8 release was measured as described under “Experimental Procedures.” Neutrophils from four donors were tested, and each mean ± S.D. after subtraction of spontaneous release (0.04 ± 0.04 ng/ml) is shown. C, purified basophils (59–88%) were incubated with the indicated concentrations of hMBP (closed squares) and hMBPH (closed circles) for 30 min at 37 °C, and histamine release was quantified as described under “Experimental Procedures.” Cells from three donors were tested, and each value is the mean ± S.D. after correction for spontaneous histamine release (9 ± 3%). D, purified basophils were incubated with the indicated concentrations of hMBP (closed squares) and hMBPH (closed circles) for 30 min at 37 °C, and LTC4 release was quantified as described under “Experimental Procedures.” Cells from three donors were tested, and each value is the mean ± S.D.; spontaneous LTC4 release was undetectable. The values shown as 0 μM protein concentration in A, C, and D represent production or release due to the sodium acetate vehicle buffer for hMBP and hMBPH. Error bars not visible in the graphs are contained within the dimensions of the data symbol.
Other current approaches to gene discovery and/or differential gene expression analysis include differential display (51), suppression subtractive hybridization (52), microarray hybridization (53), and serial analysis of gene expression (54). Each technique ultimately has advantages and disadvantages. Limitations of Transcript Imaging include the requirement for a substantial quantity of starting polyadenylated mRNA, approximately 2 μg, and the extensive labor and cost involved in generating sequence data for thousands of cDNA clones. However, of the above mentioned methods only differential display (51) requires substantially less RNA, but with a corresponding loss in completeness of the gene expression profile. Alternatively, serial analysis of gene expression (50, 54, 55) may be a viable option to generate a comparable gene expression profile of both known and unknown genes with less labor and cost. However, with serial analysis of gene expression, further work to generate a more complete cDNA sequence may be necessary. Similarly, with increasing knowledge of human cDNA and genomic DNA sequences, microarray hybridization technology (53) may allow screening of whole genomes if the appropriate complementary sequences are known and available. Nonetheless, Transcript Image technology remains a valuable approach to both quantitative and qualitative assessment of gene expression in cells or tissues.

Protein Sequence Comparisons of Major Basic Proteins—Other species known to produce a protein homologous to hMBP include mouse, rat, and guinea pig (reviewed in Ref. 1). Inter estingly, guinea pigs produce two distinct MBP homologs of similar molecular weights and isoelectric points (calculated pI values of 11.7 and 11.3) (56). The presence of a second MBP gene in the mouse has also been proposed (57). Identification of a homolog of hMBP brings the number of known mammalian prepro-MBP sequences to six. However, the uniqueness of hprepro-MBP becomes apparent upon alignment of these sequences (Fig. 7).

In the signal sequence, only six amino acids are conserved throughout all the known MBPs. However, the more general signal sequence pattern of one or more positively charged amino acids followed by a continuous stretch of 6–12 hydrophobic amino acids (58) is present in hprepro-MBP as in the other forms. Therefore, it is likely, and its presence in eosinophil granule preparations and preliminary protein expression data5 confirm that hMBP can also be secreted.

In the pro section, even less identity is observed across the various homologs (Fig. 7). However, the total number of acidic residues (Asp or Glu) for each of the pro sections remains in the range of 25–32, with hMBP’s pro section containing 28 (Table 1). However, the total number of acidic residues (Asp or Glu) for each of the pro sections remains in the range of 25–32, with hMBP’s pro section containing 28 (Table 1).

4 The deduced amino acid sequence of hprepro-MBP has been scanned against the nonredundant GenBank Protein Data Bank/ Swiss-Prot/Pfam database, and the following sequences were found to be significantly homologous: human eosinophil granule major basic protein precursor, gi 119239, sp P13727, or Y00809; mouse major basic protein, gi 1109659 or L46768; guinea pig eosinophil granule major basic protein 1 precursor, gi 119238, sp P22692, or D90251; guinea pig eosinophil granule major basic protein 2 precursor, gi 544241, sp P35709, or D00817; rat eosinophil major basic protein precursor gi 2143721, pir S68150, or D50568, similarity to C-type lectin domains, gi 1526393 or U59752.

5 D. A. Plager and J. G. Gleich, unpublished data.
conserved in all previously known pro-MBPs. Therefore, it appears that hpro-MBPH is substantially less glycosylated than any of the other homologs. The possible consequence(s) of this remains to be determined, but in general glycosylation can be involved in protein trafficking, protein folding, resistance to proteolysis, and molecular recognition/binding.

In the MBP section, the greatest degree of homology is found with 42 of the approximately 117 amino acids being identical. However, in contrast to the pro section of hpro-MBPH, where homology is low but net negative charge is conserved, the highly homologous MBP section of hMBPH has a net positive charge, which is substantially diminished compared with the other MBPs. Specifically, the net charge of hMBPH at neutral pH is near 8+ while hMBP and the other MBPs have about 16+ to 20+ net charge (Table I and Fig. 7). However, strict conservation of most basic amino acids appears to be of minimal importance. With inclusion of hMBPH in the amino acid sequence alignment, only a single basic residue, arginine at position 234, is strictly conserved. Moreover, even without inclusion of hMBPH in the alignment, only an additional two basic residues, arginine at positions 154 and 241, are conserved among the remaining five MBPs. Overall, with regard to basicity, hMBPH appears to be the most divergent form of the known MBPs.

The 2-fold reduction in net positive charge of hMBPH appears to reduce its ability to inhibit cellular protein synthesis, to induce neutrophil IL-8 production, and to induce basophil histamine and LTC₄ release compared with hMBP (Figs. 5 and 6). The more basic granule proteins, ECP and eosinophil peroxidase, also demonstrated greater protein synthesis inhibition than the less basic EDN. This suggests the importance of a molecule’s net positive charge in this cytotoxicity assay. However, arguing against net positive charge as the sole determinant of hMBP’s activities are the following: 1) comparable induction of neutrophil superoxide production by hMBP and hMBPH (Fig. 6); 2) the lack of cytotoxicity by EDN (Fig. 5), a molecule of similar mass and apparently similar net charge to hMBPH; and 3) the inability of ionic strength changes on some hMBP activities and by the isolation of a second human MBP gene and protein will verify the presence of a second MBP-like molecule in humans, as in guinea pigs. In general, the pre, pro, and MBP sections and the two disulfide linkages within the MBP section are conserved. The tissue expression profile of hprepro-MBP is similar to that of hpro-MBP, except for the expression of hpro-MBP by the placenta. Human MBHP, however, is much less basic than the other known MBPs and this tended to diminish, but not abolish, hMBP’s activity in cytotoxicity and cytostimulatory assays as compared with hMBP. Finally, the isolation of a second human MBP gene and protein will allow assessment of comparative gene promoter structure (39), evolution, protein processing and cellular localization, and protein structure-function relationships between the human eosinophil granule MBPs.

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