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Advances in Brief

Genomic Structure and Genetic Mapping of the Human Neutral Cysteine Protease Bleomycin Hydrolase[1]

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Abstract

Bleomycin hydrolase (BH) is the only known eukaryotic enzyme that inactivates the widely used antineoplastic agent bleomycin (BLM) and is a primary candidate gene for protection against lethal BLM-induced pulmonary fibrosis and for BLM resistance in tumors. Human *BH* was found to exist as a single gene that was mapped to chromosome 17 using National Institute of General Medical Sciences human/rodent hybrid mapping panels and localized to 17q11.1–11.2 by linkage analysis using the Centre d'Etude du Polymorphisme Humain reference database. The human *BH* gene consisted of 11 exons ranging in size from 69–198 bp separated by introns of approximately 1 kb, reflecting the archetypal genomic structure of the cysteine protease family. A polymorphic site was identified in the eleventh exon at bp 1450 encoding either valine or isoleucine. These findings provide essential tools required to define the role of BH in BLM-induced pulmonary fibrosis and BLM resistance in tumors.

Introduction

BLM[3] is an essential component of combination chemotherapy regimens (1, 2). Despite the importance of BLM for curative therapies, low doses of BLM induce pulmonary toxicity in approximately 3–5% of patients, and cumulative doses greater than 450 mg induce potentially lethal pulmonary toxicity in up to 10% of patients (1). The molecular basis for this untoward effect is not established, and accurate methods to screen patients for sensitivity before treatment are unavailable. Recent studies indicate a genetic basis for susceptibility to BLM-induced pulmonary fibrosis (3). BH is the only known eukaryotic enzyme that can inactivate BLM (1), and there is considerable evidence that BH can protect pulmonary tissue from BLM-induced lung injury (1, 4). Deletion of BH in yeast cells results in increased sensitivity to BLM (5), and expression of yeast BH in mammalian cells results in resistance to BLM (6). Mammalian BH has been purified to homogeneity, and its substrate specificity, inhibitor sensitivity, and deduced catalytic domains suggest its membership in the cysteine protease superfamily that includes the calpains, cathepsins, and the prototype, papain (7). Human BH cDNA encodes a 455-amino acid polypeptide that is highly conserved among species with 40% identity to the yeast BH homologue Gal6 (8). Human BH is expressed in many tissues with elevated expression levels found in the testis, skeletal muscle, and pancreas and low expression levels seen in the colon and peripheral blood leukocytes (8). Based on crystal structure analysis, yeast BH is characterized as a homohexamer that resembles a 20S proteasome with a prominent central channel housing the conserved cysteine protease active sites (9). Yeast BH is also reported to bind DNA and to act as a repressor of the Gal4 regulatory system (10). Thus, BH, which has been conserved during evolution, is likely to have important physiological functions in human cells including its key role in drug metabolism. We now report the genetic mapping of the human *BH* gene and its overall genomic organization, which corroborates membership in the cysteine protease superfamily.

Materials and Methods

Characterization of Human BH Genomic Structure. Amplification primers (Fig. 1) from adjacent exons were designed using the human cDNA sequence (8) and intron-exon boundary data from the 5′ end of the mouse *BH* gene.[4] Intronic regions were amplified from human genomic DNA using long-distance PCR (Elongase; Life Technologies, Inc., Gaithersburg, MD) and sized on ethidium bromide-stained 1% agarose gel with a 1-kb ladder molecular size marker (Life Technologies, Inc.). Confirmation of the identity of the PCR products and determination of intron-exon boundaries were performed by dye-labeled terminator direct cycle sequencing of the PCR products (11) using the DNA sequencing kit (Applied Biosystems, Foster, CA). Sequencing products were analyzed on an ABI Prism 377 automatic DNA sequencer (Applied Biosystems) and assembled using sequencer v.3.0 (Gene Codes Corp., Ann Arbor, MI). The intron-exon boundary between intron 6 and exon 6 was determined from the homologous region of the cloned mouse gene.[4]

Chromosomal Mapping of the Human *BH* Gene. Preliminary localization of the human *BH* gene was obtained using PCR-based analysis of NIGMS monoclonal hybrid mapping panel 2 (Coriell Institute for Medical Research, Camden, NJ) with primers BHB11 and BHB12 described in Fig. 1. Amplification was performed in a DNA thermal cycler 480 (Perkin-Elmer Corp., Foster City, CA) in a final volume of 25 μl containing 500 ng of genomic DNA, 0.5 unit of Taq polymerase (Life Technologies, Inc.), 1.5 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 240 nM each primer, and 7% DMSO. Thermal cycling comprised 30 cycles of 1-min denaturation at 94°C, 30-s annealing at 58°C, and 1-min extension at 72°C. Results from monoclonal hybrid mapping panel 2 were confirmed by analysis of NIGMS human/rodent hybrid mapping panel 1 (Coriell Institute for Medical Research). Panel 1 was probed with the BHB11/BHB12 primer pair as well as a second primer pair comprised of BHB11 and BHC12 using long-distance PCR (Elongase; Life Technologies, Inc.). Regional localization of the *BH* gene was performed using the NIGMS chromosome 17 regional mapping panel (Coriell Institute for Medical Research).

Genetic Mapping of Human BH. Based on the report of a possible polymorphism at bp 1450 (8), exon 11 was amplified for SSCP analysis using primers BHZ11 and BHZ12 (Fig. 1) with the same reaction conditions as described for primers BHB11 and BHB12. The PCR products were screened by a modified SSCP analysis (12) using 4–20% Tris-borate EDTA precast gels (Novex, San Diego, CA) run on a vertical electrophoresis unit (Mighty Small II Vertical Electrophoresis Unit; Hoefer, San Francisco, CA) for 2.5 h with at 20 mA with a temperature gradient of −7°C–20°C (Programmable Temperature Controller; Polyscience, Niles, IL). Gels were visualized by staining with SYBR Green II (Molecular Probes, Eugene, OR) for 20 min followed by inspection using the still video imaging system (Eagle Eye II; Stratagene, La Jolla, CA). Confirmation of the A1450G polymorphism and genotyping was performed by direct cycle sequencing of the PCR products. The human *BH* gene was genetically mapped by linkage analysis using the 10 informative

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[3] The abbreviations used are: BLM, bleomycin; BH, bleomycin hydrolase; SSCP, single-strand conformational polymorphism; NIGMS, National Institute of General Medical Sciences; ABI, Applied Biosystems Incorporated; CHLC, Cooperative Human Linkage Center; LOD, log of odds; CEPH, Centre d'Etude du Polymorphisme Humain.

[4] T. P. G. Calmels, D. R. Schwartz, and J. S. Lazo, unpublished observations.

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Fig. 1. Intron-exon boundaries of the human *BH* gene. Exons are indicated in *bold uppercase letters*. Fully conserved consensus sequences at the intron-exon junction sites are *italicized*. Primers are *underlined*, with the primer names shown *below* the underlined segment. (+) or (−) next to the primer name indicates forward (+) or reverse (−) primer. Primer pairing is indicated by a number in parentheses next to primer name, *e.g.*, BHA11(+,1) and BHB12(−,1) form primer pair 1.

GGCGCCATGAGCAGCTCGG……cctgtttggtctcccaac*ag*GACTGAATTCGGAGAA
BHA11(+, 1) BHB11(+, 2)

GGTAGCTGCTCTGATACAGAAACTGAATTCCGACCCCCAGTTCGTACTTG

CCCAGAATGTCGGGACCACCCACGACCTGCTGGACATCTGTCTGAAGCG

GGCCACGGTGCAGCGCGCGCAGCATGTGTTCCAGCACGCCGTGCCCCAG

GAGGGCAAGCCAATCACCAACCAGAAGAGCTCAG*gt*gtgggctgccacttctcc......gat
BHB12(-, 1)

gtgctttcttgtttc*ag*GGCGATGCTGGATCTTTTCTTGTCTGAATGTTATGAGGCTT
BHC11(+, 3)

CCATTCATGAAAAAGTTAAATATTGAAGAATTTGAGTTTAGCCAATCTTA
BHC12(-, 2)

CCTGTTTTTTTGGGACAAG*gt*atgggacagatctggcaa.....taactttctttactcttt*ag*GTTGAAC

GCTGTTATTTCTTCTTGAGTGCTTTTGTGGACACAGCCCAGAGAAAGGAG
BHD11(+, 4)

CCTGAGGATGGGAGGCTGGTGCAGTTTTTGCTTATGAACCCTGCAAATG
BHD12(-, 3)

ATGGTGGCCAATGGGATATGCTTGTTAATATTGTTG*gt*aaaagttctttctttaat.....tag

aatttgttttctttt*ag*AAAAATATGGTGTTATCCCTAAGAAATGCTTCCCTGAATCTT
BHE11(+, 5) BHE12(-, 4)

ATACAACAGAGGCAACCAGAAGGATGAATGATATTCTGAATCACAAG*gt*ac

aatatatgaagcaggg.….tgaataagtttctgtcat*ag*ATGAGAGAATTCTGTATACGACTGCGA

ACCTGGTACACAGTGGAGCAACGAAAGGAGAAATCTCGGCCACACAGGA
BHF12(-, 5)

CGTCATGATGGAGGAG.....ATATTCCGAGTGGTGTGCATCTGTTTGGGTAA
BHG11(+, 6)

TCCACCAGAGACATTCACCTGGGAATATCGAGACAAAGATAAAAATTATC

AGAAAATTGGCCCCATAACACCCTTGGAGTTTTACAGGGAACATGTCAAG

CCACTCTTCAATATGGAAGATAAG*gt*tggtgaatggcactggta.....tacttaacaatgtggctgtagt

acttagcttgtaggcagagatgatgagcttgatggaaacatgaacctagaggagagctgtgtgttgcttgacattcctggttttgt

gccctaattaatgtgttcttgatcttgat*ag*ATTTGTTTAGTGAATGACCCTAGGCCCCAGCAC

AAGTACAACAAACTTTACACAGTGGAATACTTAAGCAATATGGTTGGTGG
BHH12(-, 6)

GAGAAAAACTCTATACAACAACCAGCCCATTGACTTCCTGAAAAAGATGG
BHH11(+, 7)

TTGCTGCCTCCATCAAAGATGGAGAG*gt*tggtattgtttctgtattttgcacattggggttaaaacatct

tttacttctttggtataataggaaaatggggtttatgttgactttcttcctctttcttctgtttct*ag*GCTGTGTGGTTTG

GCTGTGATGTTGGAAAACACTTCAATAGCAAGCTGGGCCTCGTGACATG
BH212(-, 7)

AATCT*gt*gattgcaggaaatttaaa.....TACTATCATTCTTTTTACAGCTATGACCATGA

GTTAGTGTTTGGTGTCTCCTTGAAGAACATGAATAAAGCGGAGAGGCTG
BHI12(-, 7)

ACTTTTGGTGAGTCACTTATGACCCACGCCATGACCTTCACTGCTGTCTC

AGAGAAGGTGATCAGGATGGTGCTTTCACAAAATGGAGAGTGGAGAATT

CATGGGGTGAAGACCATGGCCACAAAGGTTACCTGTGCATGACAGATGA

GTGGTTCTCTGAGTATGTCTACGAGTGGTGGTGGACAGGAAGCATGTCC
BHZ11(+, 8)

CTGAAGAGGTGCTAGCTGTGTTAGAGCAGGAACCCATTATCCTGCCAGC

ATGGGACCCCATGGGAGCTTTGGCTGAGTGAGTGATACTGCCCTCCAGC

TCTTTCCTCCTTCCATGGAACCTGACGTAGCTGCAAAGGACAGATCCAGG
BHZ12(-, 8)

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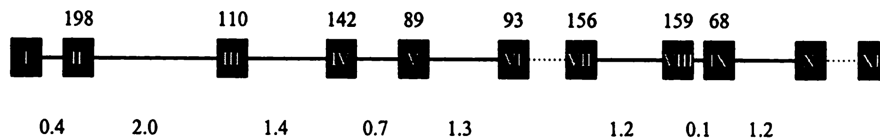
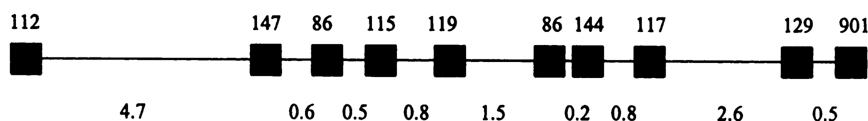
A

Human Bleomycin Hydrolase



B

Mouse Cathepsin B



Human Cathepsin L

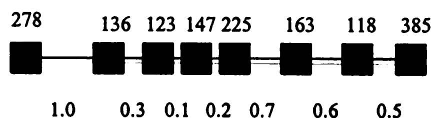


Fig. 2. Genomic structure of human BH and representative members of the cysteine protease family. Exons are indicated as *boxes*; exon size is indicated in bp *above* the figure. Introns are drawn to scale. Intronic distances are indicated *below* the figure in kb. *A*, genomic structure of human BH. The size of introns 6 and 10 has not been determined. Exon 1 contains 13 bp of coding sequence, and exons 10 and 11 together contain 337 bp of coding sequence. *B*, genomic structures of related members of the cysteine protease family. Mouse cathepsin B and human cathepsin genomic structures were from Refs. 15 and 21, respectively.

CEPH families (12, 66, 104, 1331, 1332, 1333, 1344, 1346, 1347, and 1377) that were genotyped for the exon 11 polymorphism by SSCP. Determination of the most likely map position was performed using CRI-MAP (13) with 50 chromosome 17 markers from the ABI/CHLC/CEPH v0.8 integrated sex-averaged map.

Results

Genomic Structure of the Human *BH* Gene. Eleven exons have been identified encoding human BH using long-distance PCR. The exons ranged in size from 69–198 bp, and intervening introns averaged 1 kb but ranged in size from 0.1–2.0 kb. The GC content of the exons ranged from 36% in exon 3 to 59% in exon 2. The human *BH* gene spanned approximately 10 kb (Fig. 2*A*); introns 6 and 10 were resistant to PCR amplification, perhaps due to large size or secondary structure in the region. The conserved catalytic cysteine (Cys^{73}) was contained in exon 3. Comparisons of the genomic structure of human BH with other members of the cysteine protease papain superfamily revealed a conserved architecture comprising small 100–200-kb exons separated by introns of approximately 0.5–1 kb (Fig. 2*B*).

Mapping of the Human *BH* Gene. PCR-based analysis of NIGMS human/rodent hybrid mapping panel 2 resulted in localization of the human *BH* gene to chromosome 17 (data not shown). PCR analysis of NIGMS human/rodent hybrid mapping panel 1 confirmed the chromosomal assignment (data not shown). Analysis of the NIGMS chromosome 17 regional mapping panel localized the human *BH* gene to the region of 17p11.1–17q11.2 (Fig. 3).

Genetic Mapping of the Human *BH* Gene. An A1450G polymorphic site suggested previously from human BH cDNA (8) was confirmed in exon 11, leading to a protein with either Val^{443} or Ile^{443}. The two variants were clearly discernible by SSCP analysis (Fig. 4). The allele frequencies were 0.61 for the A allele and 0.39 for the G allele based on 35 CEPH family grandparents. Two-point linkage analysis of the human *BH* gene with 50 chromosome 17 markers from the ABI/CHLC/CEPH v0.8 integrated sex-averaged map resulted in a LOD score of 4.96 at $\theta = 0.14$ with D17s975 and a LOD score of 4.45 at $\theta = 0.15$ with D17s1299. Multipoint analysis yielded the best-order map (1000:1 odds) shown in Fig. 5. Restriction endonuclease fingerprinting was used to try to localize other polymorphic regions in the human *BH* gene, but to date, none have been detected (data not shown).

Discussion

The human BH genomic structure and chromosomal localization have been determined. The human BH genomic structure was remarkably similar to that of other related members of the cysteine protease papain superfamily, which includes at least 12 other unique mammalian cysteine proteases (14). Among the papain superfamily, the lysosomal cathepsins, cytoplasmic calpains, and BHs are found to share conserved active site residues and are believed to be derived from an ancestral protease that has evolved through gene duplication (14). Our results corroborate this suggestion. Of the related cysteine proteases, cathepsin H displays *in vitro* peptidase action similar to BH *in vitro* (7) but does not share any remarkable amino acid sequence homology with human BH other than in the catalytic regions. In general, the genomic structures that have been characterized among members of the cysteine protease family show no obvious correspondence between exon divisions and structural or functional units (15); however, an intron-exon junction frequently has been noted near the active site cysteine (16). Qian *et al.* (15) hypothesize that the ancestral cysteine protease gene has undergone exon shifting during the evolutionary process. Consistent with this notion, the active site cysteine

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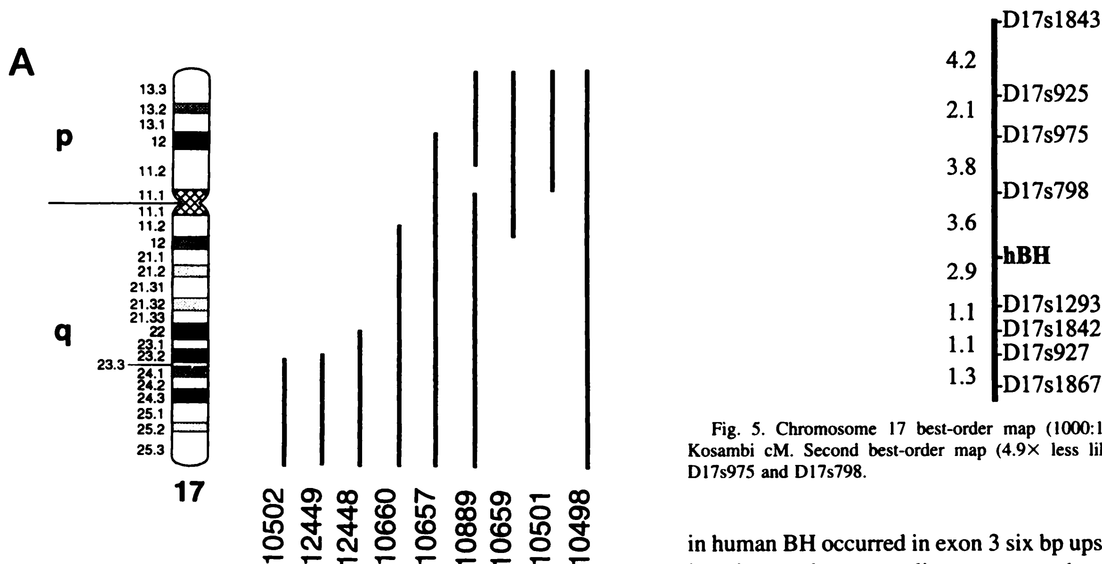

Fig. 5. Chromosome 17 best-order map (1000:1 odds). Map distances noted in Kosambi cM. Second best-order map (4.9× less likely) places human BH between D17s975 and D17s798.

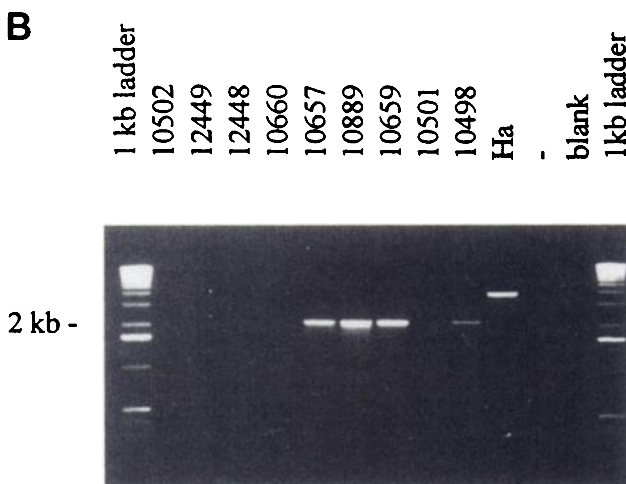

Fig. 3. NIGMS chromosome 17 regional mapping panel. *A*, ideogram of the regional mapping panel with deletion hybrids (courtesy of Dr. J. Beck, Coriell Institute for Medical Research). *B*, PCR analysis of the chromosome 17 regional mapping panel. The panel was probed with primers BHB11 and BHC12, which yielded a 2-kb amplicon from human genomic DNA. PCR products were analyzed on an ethidium bromide-stained 1% agarose gel.

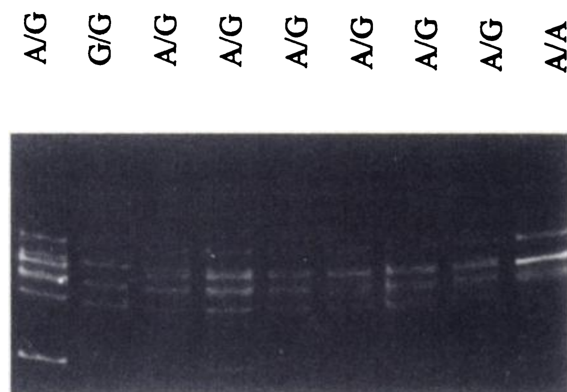

Fig. 4. SSCP genotyping of the A1450G polymorphism of human BH in CEPH families. SSCP analysis of the 160-bp PCR amplicon of primers BHZ11 and BHZ12 was performed as described in "Materials and Methods." A SYBR Green II-stained 4–20% polyacrylamide gel was loaded with samples from the following CEPH family members: 6608, 6609, 6610, 6611, 6612, 10401, 10402, 10403, and 10404. Genotype is indicated *above* each lane (*A/A*, *A/G*, or *G/G*).

in human BH occurred in exon 3 six bp upstream from the intron-exon junction, perhaps revealing a preserved exon structure encompassing a highly conserved catalytic region of the papain superfamily. The polymorphism found in exon 11 confirmed that noted by Brömme *et al.* (8) during the sequencing of the human BH cDNA. Due to the conserved substitution of a valine for an isoleucine, Brömme *et al.* (8) argue that the polymorphism is likely not to have any functional consequence.

The human *BH* gene was mapped to chromosome 17 by several complimentary methods. Human *BH* seemed to be a single-copy gene, based on all data from mapping and genomic structural analysis. Related cysteine protease genes are similarly found as single-copy genes without any apparent clustering on individual chromosomes. The best-order map location for human BH derived from genetic mapping using the CEPH database was in agreement with localization of the human *BH* gene using NIGMS human rodent hybrid mapping panels. The best-order map places human BH between markers D17s798 and D17s1293. D17s145, which lies 1 cM from D17s798, has been localized to 17q11.1–17q11.2 (17), consistent with our positioning using the chromosome 17 regional mapping panel. LOD scores for markers in the 12.2-Kosambi cM region between D17s975 and D17s1299 (ABI/CHLC/CEPH v0.8 integrated sex-averaged map) were uninformative in the two-point linkage analysis due to the lack of informative meiosis.

The localization of human BH on chromosome 17 implies that the mouse homologue should be situated on mouse chromosome 11, due to the high degree of synteny between the two species (18). Interestingly, recent linkage studies with inbred murine strains that display differential BLM sensitivity suggest a locus that is linked to sensitivity to BLM-induced pulmonary fibrosis at D11Mit35 (3).[5] D11Mit35 is near to the mouse gene *CRYBA* (19); *CRYBA1*, the human homologue, maps to 17q11.2–17q12 (20).

The genomic structure data and chromosomal mapping of BH provide a valuable resource for additional studies on the role of human BH in the etiology of BLM-induced pulmonary fibrosis and BLM therapy resistance. Moreover, the genomic architecture of human BH supports the role of this enzyme as a member of the diverse cysteine protease family.

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[5] C. Haston and E. Travis, personal communication.

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providing the initial mouse genomic sequence. We also appreciate the thoughtful comments of I. Lefterov and G. Homanics concerning this manuscript.

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