Bleomycin Hydrolase: Molecular Cloning, Sequencing, and Biochemical Studies Reveal Membership in the Cysteine Proteinase Family†

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ABSTRACT: Bleomycin (BLM) hydrolase catalyzes the inactivation of the antitumor drug BLM and is believed to protect normal and malignant cells from BLM toxicity. The normal physiological function of BLM hydrolase is not known. We now provide evidence for its membership in the cysteine proteinase family. BLM hydrolase was purified to homogeneity from rabbit lungs, and a partial amino acid sequence was determined from a tryptic digest peptide. On the basis of this sequence a 36-mer oligonucleotide was synthesized. The 36-mer oligonucleotide probe hybridized to a single mRNA species of 2.5 kb from several species and was used to isolate an 832-bp cDNA insert from a λgt11 rabbit liver cDNA library. This insert encoded the tryptic digest peptide previously identified in rabbit lung BLM hydrolase by amino acid sequencing. Analysis of the predicted amino acid sequence coded by the 832-bp BLM hydrolase cDNA fragment indicated no significant homology with any currently known proteins except for a 15 amino acid portion, which displayed remarkable homology with the active site of cysteine proteinases. Within this active-site region, 10 of the amino acid residues of papain and 9 of aleurain, cathepsin H, and cathepsin L were identical with those of rabbit liver BLM hydrolase. The catalytic cysteine of thiol proteinases was also conserved in BLM hydrolase, and cysteine proteinase specific inhibitors, such as E-64, were found to be potent inhibitors of BLM hydrolase activity. Furthermore, bleomycin hydrolase exhibited cathepsin H like enzymatic activity. Bleomycin hydrolase had, however, no significant cathepsin B or L activities. These results demonstrate that BLM hydrolase is a new member of the cysteine proteinase family with cathepsin H like activity.

Bleomycin (BLM),[1] a glycopeptide produced by *Streptomyces verticillus*, is an important component of several combination chemotherapeutic regimens used to treat human malignancies (Lazo et al., 1987; Lazo & Sebti, 1989; Sebti & Lazo, 1988; Carter, 1985; Bennett & Reich, 1979). One unique feature of BLM is its lack of significant hepatic, renal, and bone marrow toxicities (Umezawa, 1971), untoward effects frequently associated with other anticancer drugs. There are, however, two major obstacles that limit the use of BLM: tumor resistance (Lazo et al., 1987; Lazo & Sebti, 1989; Sebti & Lazo, 1988) and BLM-induced pulmonary toxicity (Lazo et al., 1987; Bennett & Reich, 1979). The susceptibility of both normal and malignant tissue to BLM-induced toxicity appears to depend on the levels of BLM hydrolase, an enzyme that inactivates BLM by hydrolyzing the alanine carboxyamide moiety (Lazo et al., 1987; Lazo & Sebti, 1989; Sebti & Lazo, 1988; Umezawa, 1971; Umezawa et al., 1972, 1974). Tumors in vivo (Umezawa et al., 1972; Umezawa, 1974) and cells in culture (Akiyama et al., 1981; Ueda et al., 1984) that maintain high levels of BLM hydrolase activity are resistant to BLM, whereas those that respond to BLM have low levels of this enzyme activity. Similarly, lungs are sensitive to BLM-induced pulmonary injury and maintain lower levels of BLM hydrolase activity compared to other organs (Lazo & Humphreys, 1983).

Recently, we purified to homogeneity and characterized this protective enzyme from rabbit lungs (Sebti et al., 1987, 1989; Sebti & Lazo, 1987). The pure enzyme has a native molecular mass of 250 kDa and consists of 5 identical subunits of 50 kDa (Sebti et al., 1987). BLM hydrolase is strongly inhibited by leupeptin, puromycin, NEM, and divalent cations (Sebti et al., 1987). Nishimura et al. (1987) also independently purified BLM hydrolase from rabbit liver and reported similar biochemical properties to the rabbit lung enzyme.

The normal physiological role of BLM hydrolase is unknown. Sebti and Lazo (1987) demonstrated that BLM hydrolase is not an aminopeptidase as previously suspected (Umezawa et al., 1974). The present paper describes the molecular cloning and sequencing of a cDNA fragment encoding BLM hydrolase. On the basis of the deduced amino acid sequence coded by this nucleotide sequence and enzyme activity studies, BLM hydrolase is identified as a new member of the cysteine proteinase family with cathepsin H like activity.

Materials and Methods

Amino Acid Sequence Analysis. Rabbit lung BLM hydrolase was purified to homogeneity as described previously (Sebti et al., 1987). The purified enzyme (approximately 25 μg) was subjected to cleavage by trypsin, and the resulting peptides were separated by reverse-phase HPLC on a Aquapore column (25 cm × 1 mm) (Brownlee, Foster City, CA)

† The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02866.

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[1] Abbreviations: PAGE, polyacrylamide gel electrophoresis; HSLC, high-speed liquid chromatography; BLM, bleomycin; NEM, *N*-ethylmaleimide; Z-Phe-Arg-NMec, (benzyloxycarbonyl)phenylalanylarginine 4-methyl-7-coumarylamide; Z-Arg-Arg-NMec, (benzyloxycarbonyl)arginylarginine 4-methyl-7-coumarylamide; Arg-NMec, arginine 4-methyl-7-coumarylamide; N-Mec, 7-amino-4-methylcoumarin; E-64, 1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane.

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(Stone & Williams, 1986). The amino acid sequence was determined by using a gas-phase amino acid sequencer (Model 470A, Applied Biosystems).

Oligonucleotide Probe Design. Sequencing of peptide 24 (Figure 1) resulted in the determination of its 25 amino acid sequence (Figure 1). On the basis of this sequence, a 36-mer oligonucleotide was designed by using the mammalian codon usage frequencies table as described by Lathe (1985). The 36-mer oligonucleotide was then synthesized with a DNA synthesizer (Model 380B, Applied Biosystems).

Northern Blot Analysis. Total RNA was isolated by the guanidinium isothiocyanate/CsCl method (Chirgwin et al., 1979) or by a single-step method (Chomczynski & Sacchi, 1987). Poly(A+) was then purified by oligo(dT)–cellulose column chromatography (Davis et al., 1986). Electrophoresis of RNA was performed in formaldehyde-containing agarose gels followed by transfer to nylon membranes (Davis et al., 1986). The membranes were dried and prehybridized for 2 h at 42 °C in 6× SSC, 5× Denhardt's solution [0.1% each Ficoll 400, poly(vinylpyrrolidone), and bovine serum albumin], 1% SDS, and 50 μg/mL denatured salmon sperm DNA (0.2 mL/cm^2 of membrane). The membranes were then hybridized over 14 h at 32 °C with the 36-mer probe or 48 h at 39 °C with the BLM hydrolase cDNA in 50 μL/cm^2 of membrane with the above solution in 50% formamide and ^{32}P-labeled BLM hydrolase cDNA or 36-mer oligonucleotide probe (10^6 cpm/mL). The BLM hydrolase cDNA probe was labeled by the random oligonucleotide primed extension method of Feinberg and Vogelstein (1983), and the 36-mer was 5′-end-labeled with T_4 polynucleotide kinase (Davis et al., 1986). Following hybridization, the membranes were washed twice for 15 min first in 5× SSC and 1% SDS at room temperature and then in 1× SSC and 1% SDS at 50 °C followed by one final wash at room temperature in 0.1× SSC and 0.1% SDS. Autoradiography was then performed by exposing X-ray film to the membranes at −70 °C.

cDNA Library Screening. The 36-mer was used to screen a rabbit liver λgt11 cDNA library (a gift of Dr. R. Tukey, University of California, San Diego, CA) (Quattrochi et al., 1986). About 750000 plaques were transferred to nylon membranes and hybridized with the 36-mer oligonucleotide probe as described for Northern blot analysis. One positive clone was purified and expanded (Davis et al., 1986). *Eco*RI digestion of phage DNA from this clone resulted in two small cDNA fragments, which were used to screen another rabbit liver λgt11 cDNA library. With this second cDNA library (kindly provided by Dr. P. Kroon, Merck Sharpe and Dohme Research Laboratories, Rahway, NJ) (Kroon et al., 1986) a higher percentage of recombinant phage containing larger inserts were found. Approximately 300000 plaques were analyzed and 11 positive clones were identified. The BLM hydrolase cDNA inserts were ligated into the *Eco*RI site of M13 mp18 (Yanisch-Perron et al., 1985) for sequencing by standard dideoxy methodology (Sanger et al., 1977).

Inhibition Studies. BLM hydrolase was partially purified from rabbit lung by homogenization of the lungs and chromatography of the postmicrosomal supernatant with DEAE-Sephacel followed by phenyl-Sepharose chromatography as described previously (Sebti et al., 1987). Aliquots (20 μL, 1.7 mg/mL) of this BLM hydrolase preparation were preincubated with 40 μM of the inhibitor E-64 (Barrett et al., 1982) for 5 min at room temperature. The samples were then incubated with BLM A_2 (60 μM) for 60 min at 37 °C and assayed for BLM hydrolase activity by HSLC as described previously (Sebti et al., 1987; Sebti & Lazo, 1987).

Enzyme Assays for Cathepsins B, H, and L. The methods used here were those of Barrett (Barrett, 1980; Barrett & Kirschke, 1981) with slight modifications (Barrett et al., 1982). Rabbit lung BLM hydrolase was purified to homogeneity as described previously (Sebti et al., 1987). Aliquots of the pure enzyme (50 ng/μL) were diluted to 500 μL with 0.1% Brij 35 solution (Sigma, St. Louis, MO) and added to 250 μL of stock buffer (352 mM KH_2PO_4, 48 mM Na_2HPO_4, 4 mM Na_2EDTA, and 8 mM dithiothreitol). The assays were started by adding 250 μL of the various substrates (20 μM in 0.1% Brij 35) and incubating the reaction mixtures for 30 min. For cathepsin H, the substrate used was Arg-NMec at 30 °C and pH 6.8 (Barrett & Kirschke, 1981). Z-Phe-Arg-NMec at 30 °C and pH 5.5 was used for cathepsin L, whereas Z-Arg-Arg-NMec at 40 °C and pH 6 was used for cathepsin B (Barrett & Kirschke, 1981). The reaction was stopped with 1.0 mL of 100 mM sodium monochloroacetate in 100 mM sodium acetate, pH 4.3. Fluorescence of the liberated aminomethylcoumarin was measured in a Perkin-Elmer fluorometer at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. The nanomoles of products generated by BLM hydrolase were calculated from a standard curve of 7-amino-4-methylcoumarin.

Results and Discussion

The involvement of BLM hydrolase, a BLM-inactivating enzyme, in tumor resistance and in the protection of normal tissue from BLM toxicity was suggested soon after the discovery of this drug two decades ago (Lazo et al., 1987; Lazo & Sebti, 1989; Sebti & Lazo, 1988). These roles for BLM hydrolase have not been established in part due to difficulties in purifying and characterizing BLM hydrolase. Furthermore, structural information about BLM hydrolase is essential in determining the normal physiological role of this enzyme and its involvement in the protection of normal and malignant tissues from BLM toxicity. Recently, we (Sebti et al., 1987) and Nishimura et al. (1987) purified to homogeneity and characterized this enzyme from rabbit lung and liver, respectively. We now describe the molecular cloning, sequencing, and biochemical studies of this enzyme that reveal its membership in the cysteine proteinase family.

Amino Acid Sequence and Oligonucleotide Probe Design. Rabbit lung BLM hydrolase was purified to homogeneity as described previously (Sebti et al., 1987). The pure enzyme migrated as a single band of 50 kDa in NaDodSO$_4$/PAGE (Figure 1A). Direct sequencing of BLM hydrolase failed due to a blocked amino terminus. The protein was then subjected to digestion with trypsin. The tryptic digest peptides were then resolved and purified by reverse-phase HPLC (Figure 1B) as described under Materials and Methods. Peptide peak 24, which contained the most UV-absorbing material, was subjected to amino acid sequence analysis and gave the 25 amino acid sequence shown in Figure 1C. A 36-mer oligonucleotide probe was designed and synthesized on the basis of the sequence of 12 amino acids of the carboxy terminus of peptide 24 (Figure 1C) as described under Materials and Methods. This probe was then ^{32}P-end-labeled and used in Northern blot analyses of mRNA from several species (Figure 2). The 36-mer oligonucleotide hybridized to a single 2.5-kb mRNA from human tumor cells (lane 1), lungs from BALB/c and C57BL/6 mice (lanes 2 and 3), rabbit lungs and livers (lanes 4 and 5), livers and kidneys of bullfrog (lanes 6 and 7), chicken (lanes 8 and 9), and rat (lanes 10 and 11), and baboon liver (lane 12). Thus, the probe was specific for a single message of the size sufficient to code for the BLM hydrolase protein subunit.

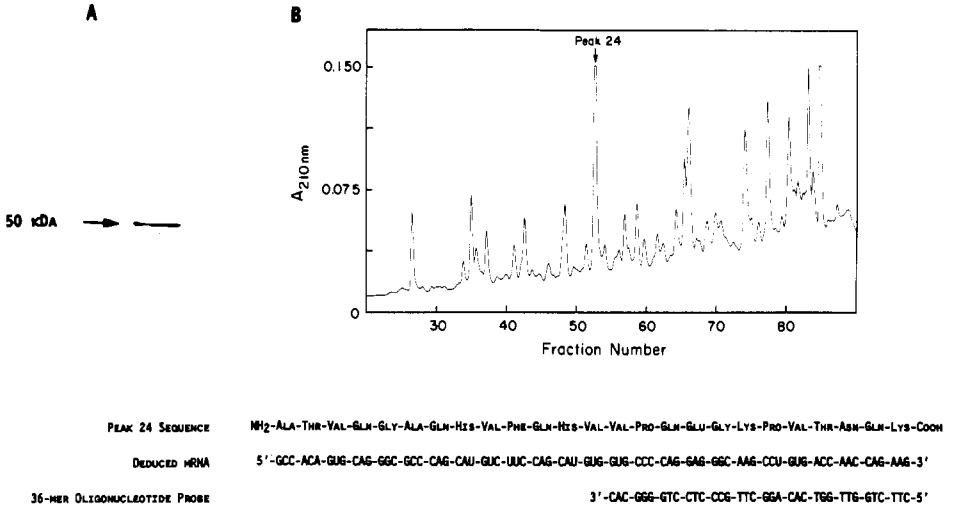

FIGURE 1: Oligonucleotide probe design. (A) BLM hydrolase was purified as described previously (Sebti et al., 1987), and 10 μg of the final enzyme preparation was electrophoresed on a 10% SDS–PAGE and stained with Coomassie blue. The arrow designates the position and apparent molecular mass of the BLM hydrolase subunit. (B) Reverse-phase HPLC profile of the tryptic digest peptides of BLM hydrolase was carried out as described under Materials and Methods. (C) Amino acid sequence of peak 24 from the tryptic digest shown in (B), its deduced mRNA sequence, and the corresponding 36-mer oligonucleotide probe sequence.

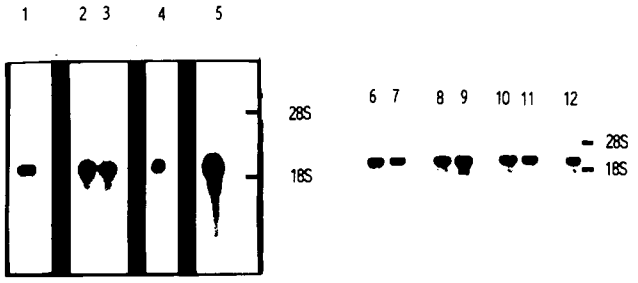

FIGURE 2: RNA blot analysis with 36-mer probe. Poly(A^+) RNA was isolated from various sources, electrophoresed, transferred to nylon filters, and probed with 5′-end-labeled 36-mer oligonucleotide as described under Materials and Methods. (1) Human leukemia HL-60 cells; (2) BALB/c lung; (3) C56BL/6 lung; (4) rabbit lung; (5) rabbit liver; (6 and 7) livers and kidneys from bullfrog, respectively; (8 and 9) livers and kidneys from chicken, respectively; (10 and 11) livers and kidneys from rat, respectively; (12) baboon liver. The positions of 28S and 18S ribosomal RNA are indicated. Approximately 3 μg was loaded on each lane.

BLM Hydrolase cDNA Isolation and Sequencing. The 36-mer oligonucleotide was then used to screen a rabbit liver λgt11 cDNA library as described under Materials and Methods. One positive clone was isolated. Upon digestion with *Eco*RI, this clone yielded two small cDNA fragments, which were then used to screen a second rabbit liver λgt11 cDNA library, yielding a clone containing a cDNA insert of approximately 800 bp. *Eco*RI digestion of the phage DNA from this clone resulted in two cDNA fragments. The larger fragment of approximately 500 bp hybridized to the 36-mer, whereas the smaller fragment of approximately 300 bp did not (data not shown). Both fragments were then sequenced as described under Materials and Methods. Figure 3 shows the nucleotide sequence of the entire 832-bp fragment and its deduced amino acid sequence. This fragment contains only one open reading frame that had the coding region of the amino acid sequence of peptide 24 used for the probe design (Figure 1C), thereby demonstrating that the 832-bp cDNA of the insert encodes BLM hydrolase. Amino acids 24–48, which are underlined in Figure 3, correspond to the amino acid sequence of peak 24 in Figure 1C, whereas nucleotides 110–146 correspond to the synthetic oligonucleotide of Figure 1C. Within the region hybridizing with the 36-mer oligonucleotide there were only three mismatches (starred bases of Figure 3).

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1    C GAC CCC CAG TTC GTG CTT GCC CAG AAT GTG GGG ACC CAC CAC GAC CTG CTG
       Asp Pro Gln Phe Val Leu Ala Gln Asn Val Gly Thr His His Asp Leu Leu  17

53   GAC ATC TGC CTG AGG AGG GCC ACG GTG CAG GGC GCG CAG CAT GTG TTC CAG CAC
     Asp Ile Cys Leu Arg Arg Ala Thr Val Gln Gly Ala Gln His Val Phe Gln His  35
                  *                 *  *
107  GTC GTG CCG CAG GAG GGC AAG CCG GTC ACC AAC GAG AAG AGC TCG GGG CGA TGC
     Val Val Pro Gln Glu Gly Lys Pro Val Thr Asn Gln Lys Ser Ser Gly Arg Cys  53

161  TGG ATC TTT TCT TGT CTG AAT GTT ATG AGA CTT CCA TTC ATG AAA AAA TTG AAT
     Trp Ile Phe Ser Cys Leu Asn Val Met Arg Leu Pro Phe Met Lys Lys Leu Asn  71

215  ATT GAA GAA TTT GAG TTT AGT CAA TCT TAT GTG TTT TTC TGG GAC AAG GTT GAA
     Ile Glu Glu Phe Glu Phe Ser Gln Ser Tyr Val Phe Phe Trp Asp Lys Val Glu  89

269  CGC TGT TAT TTC TTC TTG AAC GCT TTT GTG GAC ACA GCA CAG AAA AAG GAA CCT
     Arg Cys Tyr Phe Phe Leu Asn Ala Phe Val Asp Thr Ala Gln Lys Lys Glu Pro 107

323  GAG GAT GGG AGG CTG GTG CAG TAT TTG CTT ATG AAC CCT ACA AAC GAC GGT GGG
     Glu Asp Gly Arg Leu Val Gln Tyr Leu Leu Met Asn Pro Thr Asn Asp Gly Gly 125

377  CAG TGG GAC ATG CTT GTC AAC ATT ATT GAA AAA TAT GGT GTT GTG CCA AAG AAA
     Gln Trp Asp Met Leu Val Asn Ile Ile Glu Lys Tyr Gly Val Val Pro Lys Lys 143

431  TGC TTC CCT GAA TCA CAT ACA ACA GAG GCA AGC AGA AGG ATG AAT GAC ATT CTG
     Cys Phe Pro Glu Ser His Thr Thr Glu Ala Ser Arg Arg Met Asn Asp Ile Leu 161

485  AAT CAC AAG ATG AGA GAA TTC TGC ATA CGA CTG CGG AAC ATG GTG CAC AGT GGA
     Asn His Lys Met Arg Glu Phe Cys Ile Arg Leu Arg Asn Met Val His Ser Gly 179

539  GCA ACC AAA GCA GAA ATC TCA GCC ACG CAG GAT ACC ATG ATG GAG GAG ATA TTC
     Ala Thr Lys Ala Glu Ile Ser Ala Thr Gln Asp Thr Met Met Glu Glu Ile Phe 197

593  CGA GTG GTG TGC ATC TGT TTG GGC AAT CCA CCA GAG ACA TTC ACC TGG GAG TAT
     Arg Val Val Cys Ile Cys Leu Gly Asn Pro Pro Glu Thr Phe Thr Trp Glu Tyr 215

647  CGA GAC AAA GAT AAA AAC TAT CAG AAG ATT GGC CCC ATA ACA CCC TTG GAG TTT
     Arg Asp Lys Asp Lys Asn Tyr Gln Lys Ile Gly Pro Ile Thr Pro Leu Glu Phe 233

701  TAC AGG CAA CAC GTG AAA CCA CTC TTC AAC ATG GAA GAT AAG ATT TGT TTT GTG
     Tyr Arg Gln His Val Lys Pro Leu Phe Asn Met Glu Asp Lys Ile Cys Phe Val 251

755  AAT GAC CCT CGG CCC CAG CAC AAG TAT AAC AGA CTA TAC ACG GTA GAC TAC CTA
     Asn Asp Pro Arg Pro Gln His Lys Tyr Asn Arg Leu Tyr Thr Val Asp Tyr Leu 269

809  AGC AAT ATG GTA GGA GGG AGA AAA
     Ser Asn Met Val Gly Gly Arg Lys  277
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FIGURE 3: DNA sequence for the BLM hydrolase *Eco*RI insert and its deduced amino acid sequence. The 832-bp cDNA insert was isolated from a λgt11 rabbit liver cDNA library, digested with *Eco*RI, and sequenced as described under Materials and Methods. The positions of the derived peptide sequence of peak 24 from Figure 1C are underlined (amino acids 24–48). The starred bases represent where mismatches occurred in the design of the 36-mer oligonucleotide (bases 110–146). The boxed cysteine (amino acid 53) designates the position of the active site. The nucleotides are numbered on the left of each line, and the amino acids are numbered on the right of each line.

Expression of BLM Hydrolase mRNA. *Eco*RI digestion of the 832-bp cDNA insert of Figure 3 resulted in two fragments corresponding to nucleotides 1–500 and 501–832. The 500-bp fragment was used in RNA transfer blot analysis to determine whether BLM hydrolase is expressed in various species. Total RNA was isolated from lungs and livers of

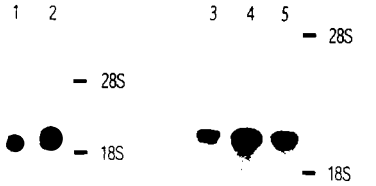

FIGURE 4: RNA blot analysis with BLM hydrolase cDNA insert. Total RNA was isolated from several sources, electrophoresed, transferred to nylon filters, and hybridized to the 500-bp *Eco*RI fragment of the BLM hydrolase cDNA insert as described under Materials and Methods. (1) Rabbit lung; (2) rabbit liver; (3) C57BL/6 mice lung, (4) liver, and (5) kidney. The positions of 28S and 18S ribosomal RNA are indicated. Approximately 10 μg was loaded on each lane.

rabbits and from lungs, livers, and kidneys of mice. The RNA was electrophoresed, transferred to nylon membranes, and hybridized to the 500-bp fragment as described under Materials and Methods. BLM hydrolase mRNA was found in rabbit lung, the organ from which BLM hydrolase was purified, and in rabbit liver, the organ from which the cDNA library was constructed (Figure 4, lanes 1 and 2). In both organs the RNA contains approximately 2500 nucleotide residues. Figure 4 also shows that mouse lungs (lane 3), livers (lane 4), and kidneys (lane 5) all expressed BLM hydrolase mRNA. Although all organs tested expressed BLM hydrolase mRNA, the levels in the lungs, the site of BLM-induced toxicity, were lower than in other organs. These results are consistent with our previous findings of the enzymatic activity levels of BLM hydrolase in these organs (Lazo & Humphreys, 1983). Our present results support the hypothesis that BLM hydrolase plays an important role in protecting organs from BLM-induced toxicity.

Furthermore, BLM hydrolase mRNA is expressed in all species that we studied, including human, rabbits, rats, mice, baboon, bullfrog, and chicken; this is consistent with previous studies (Umezawa et al., 1972, 1974; Umezawa, 1974), which showed a ubiquitous distribution of BLM hydrolase enzyme activity among tissues from different species. This indicates that the primary structure of BLM hydrolase is conserved, suggesting that this enzyme may have an important normal physiological function.

Functional Identity of BLM Hydrolase. Despite its ubiquitous nature, the normal physiological or biochemical function of BLM hydrolase remains unknown. Umezawa and co-workers (1974) suggested that this enzyme was an aminopeptidase B. Sebti and Lazo (1987), however, demonstrated unequivocally that BLM hydrolase was not related to the aminopeptidase family. Comparison of the 277 amino acid sequence deduced from the nucleotide sequence of the 832-bp BLM hydrolase cDNA fragment (Figure 3) with the sequences for proteins in the entries of the National Biomedical Research Foundation (George et al., 1986; Lipman & Pearson, 1985) revealed little homology with other known proteins except in the region containing the active site of the cysteine proteinase family. This region between amino acids 43 and 58 of the BLM hydrolase fragment (Figure 3) is shown along with the corresponding active-site region of four cysteine proteinases in Figure 5. The degree of homology between BLM hydrolase and cysteine proteinases in a 15 amino acid segment within the active-site region is significant. Figure 5 shows that within this group of cysteine proteinases there are 12 consensus residues and that 10 residues of BLM hydrolase are identical with those of papain and 9 residues are identical with those of human kidney and rat liver cathepsin H, human kidney cathepsin L, and aleurain. The active-site cysteine of these thiol proteinases is also conserved in BLM hydrolase (starred

BLEOMYCIN HYDROLASE	P	V	T	N	Q	K	S	S	G	R	C	W	I		F	S
PAPAIN	P	V	K	N	Q	G	S	C	G	S	C	W	A		F	S
CATHEPSIN H	P	V	K	N	Q	G	A	C	G	S	C	W	T		F	S
CATHEPSIN L	P	V	K	N	Q	G	Q	C	G	S	C	W	A		F	S
ALEURAIN	P	V	K	N	Q	A	H	C	G	S	C	W	T		F	S

FIGURE 5: Comparison of the amino acid sequence of BLM hydrolase with that of cysteine proteinases. Residues uniformly identical among the four cysteine proteinases and BLM hydrolase are boxed. The asterisk indicates the position of the active site of cysteine proteinases. The amino acid sequences of human kidney cathepsin H and L and the plant proteinase papain were obtained from Ritonja et al. (1988), rat liver cathepsin H from Takio et al. (1983), and the barley proteinase aleurain from Rogers et al. (1985).

C of Figure 5 and boxed Cys of Figure 3). The degree of homology between BLM hydrolase and rat liver cathepsin B (Takio et al., 1983) within the active-site region is less than with the other cysteine proteinases shown in Figure 5 but nevertheless remains high (43%).

The presence of a highly homologous region of 15 amino acids surrounding the active site of cysteine proteinases suggests that BLM hydrolase is a member of the cysteine proteinase family. To confirm this, we determined whether or not BLM hydrolase can actually perform cysteine proteinase catalytic chemistry. Thus, the ability of the pure enzyme to hydrolyze several typical cysteine proteinase substrates was tested. We have used Z-Arg-Arg-NMec, Z-Phe-Arg-NMec, and Arg-NMec, substrates that under the specific conditions described under Materials and Methods and in previous papers (Barrett et al., 1982; Barrett, 1980; Barrett & Kirschke, 1981) have been used to identify cathepsin B, L, and H, respectively. BLM hydrolase (0.25, 0.50, and 1.00 μg) was able to metabolize only one of the three substrates, Arg-NMec, and formed 70, 155, and 325 pmol of NMec/h, respectively. Thus, BLM hydrolase cleaved Arg-NMec, in a concentration-dependent fashion, but was unable to hydrolyze either Z-Arg-Arg-NMec or Z-Phe-Arg-NMec. This substrate specificity is consistent with that of cathepsin H, which is known to cleave only Arg-NMec but not Z-Arg-Arg-NMec or Z-Phe-Arg-NMec (Barrett & Kirschke, 1981). As a control experiment, we have shown that cathepsin B (Calbiochem Co., San Diego, CA) did not hydrolyze Arg-NMec but did hydrolyze Z-Arg-Arg-NMec, a behavior expected of cathepsin B (Barrett & Kirschke, 1981). The fact that BLM hydrolase has cysteine proteinase activity is consistent with our previous inhibition studies with NEM, which suggested that the active site of BLM hydrolase contains a sulfhydryl group (Sebti et al., 1987). In the present study we have further confirmed this by observing inhibition with the cysteine proteinase specific inhibitor, E-64; a concentration of 40 μM E-64 completely inhibited the conversion of BLM A_2 to its inactive metabolite BLM dA_2 by rabbit lung BLM hydrolase as described under Materials and Methods. This is consistent with the work of Nishimura et al. (1987), who also demonstrated the inhibition of rabbit liver BLM hydrolase by E-64. These results clearly indicate that BLM hydrolase contains a cysteine proteinase active site capable of performing cathepsin H catalytic activity. Differences, however, in the primary amino acid structure, molecular mass, p*I*, and pH optimum (Sebti et al., 1987; Sebti & Lazo, 1987; Nishimura et al., 1987) distinguish BLM hydrolase from the previously described cathepsin H (Ritonja et al., 1988; Takio et al., 1983).

Thus, a partial length BLM hydrolase cDNA has been cloned and sequenced. The amino acid sequence deduced from the BLM hydrolase cDNA along with the biochemical studies indicates a remarkable retention of the essential functional attributes of cysteine proteinases and demonstrates that BLM

hydrolase should be classified as a new cathepsin H like enzyme.

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