Human Bleomycin Hydrolase Binds Ribosomal Proteins[†,⊥]

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ABSTRACT: Bleomycin hydrolase (BH) is a cysteine proteinase that inactivates the anticancer drug bleomycin. Yeast BH forms a homohexameric structure that resembles a 20S proteasome and binds to single-stranded RNA and DNA. We now demonstrate that human BH (hBH) interacts and colocalizes with ribosomal proteins. Using a yeast two-hybrid system, we found hBH bound to human homologues of rat ribosomal proteins L11 and L29. The N-terminus of hBH (amino acids 14−175), which contains a catalytic Cys^{93}, was critical for the binding to L11 in the two-hybrid environment. hBH precipitated ^{35}S-labeled L11 and L29 in vitro, and hBH colocalized with L11 and L29 as determined by immunofluorescence. In addition to cytosolic bleomycin hydrolase, we found abundant bleomycin hydrolase activity associated with the ribosomal subcellular fraction by differential centrifugation. hBH was also detected by Western immunoblotting in a high-speed particulate fraction, where the majority of L11 and L29 were found. In vitro experiments showed recombinant hBH binds to Chinese hamster ovary cell microsomes. Thus, our data strongly suggest that hBH exists as both a free cytosolic and ribosome-associated protein.

Bleomycin hydrolase (BH)[1] is a 455-amino acid cysteine proteinase that confers resistance to the anticancer drug bleomycin by degrading bleomycin. BH is conserved among eukaryotes, with more than 40% amino acid identity between yeast BH (yBH) and human BH (hBH) (*1*). yBH exhibits structural similarity to a 20S proteasome and belongs to a family of self-compartmentalizing or sequestered intracellular proteases (*2*, *3*). The normal function of BH is, however, still unknown. BH clearly has demonstrable aminopeptidase activity, which is presumably responsible for bleomycin deamidation. BH may have other primary roles superseding that of an aminopeptidase. yBH has been shown to bind DNA and RNA (*4*, *5*) and is a member of the galactose regulon in yeast (*6*). Enenkel and Wolf (*7*) identified the gene for yBH as a gene encoding a protein that suppresses the in vitro phenotype of the *pre3-2* mutant yeast strain defective in one of the catalytic subunits of the yeast proteasome and devoid of Cbz-Leu-Leu-Glu-β-NA-hydrolyzing activity. We found hBH has intrinsic endopeptidase activity (*8*), while others have characterized the unusual autocarboxypeptidase and peptide ligase activity of yBH (*9*). Recently, we reported human ubiquitin-conjugating enzyme 9 (UBC9) is a partner of hBH in a two-hybrid system (*10*). hBH also colocalized and coprecipitated with UBC9, suggesting hBH may participate in posttranslational modification of the proteins. Recently, it has been suggested that one of the two isoforms of hBH (hBH_{val}) is associated with an increased risk of sporadic Alzheimer's disease in non-ApoE4 patients (*11*), although in another Caucasian population, this association was not obvious (*12*).

Previous reports in which immunofluorescence was employed suggest yBH and hBH are cytoplasmic in yeast and mammalian cells (*5*, *10*). Biochemical results indicate a significant fraction of the enzyme activity is associated with the postmicrosomal supernatant fraction (*13*). In the study presented here, we demonstrate two-hybrid and in vitro binding of hBH with ribosomal proteins L11 and L29. We also show hBH association with the ribosomal subcellular fraction from Chinese hamster ovary (CHO) cells, and binding of recombinant His-tagged hBH to isolated CHO ribosomes. Using indirect immunofluorescence in conjunction with three-dimensional image deconvolution, we found hBH colocalized with L11 and L29. Therefore, our study strongly suggests hBH exists in both free cytosolic and ribosome-bound forms.

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Two-Hybrid Screening. Yeast two-hybrid screening and analysis were performed as previously

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[⊥] This work is dedicated to the memory of Emily E. Dorrance. May we all fight cancer with her spirit.

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[1] Abbreviations: BH, bleomycin hydrolase; yBH, yeast bleomycin hydrolase; hBH, human bleomycin hydrolase; BD, binding domain; AD, activating domain; BD−hBH, GAL4BD−hBH fusion protein; AD−L11, GAL4AD−L11 fusion protein; AD−L29, GAL4AD−L29 fusion protein; L11Myc, L11−Myc epitope fusion protein; L29Myc, L29−Myc epitope fusion protein; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase.

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described (*8*). Briefly, we used the Matchmaker 2 protocol provided by Clontech (Palo Alto, CA) with the Y190 yeast strain (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4*Δ*, gal80*Δ*, cyh^{r}2, LYS2::GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*). HeLa cDNA and human liver cDNA libraries were purchased from Clontech. Y190 cotransformed with pVA3 and pTD served as positive controls. As previously described (*1*), cDNA for hBH was a gift from D. Brömme (Mount Sinai Medical Center, New York). We confirmed the expression of the hBH fusion protein by Western immunoblotting with GAL4BD monoclonal antibodies (Clontech). We determined β-galactosidase activity after transferring the yeast colonies on Whatmann filters, lysing the submerged filters in liquid nitrogen, and thawing them at room temperature. In general, β-galactosidase activity was apparent within 1 h, but the filters were incubated for 8 h.

To generate some of the recombinant expression vectors, we used hBH cDNA that contained T7 epitope sequences (Novagen, Madison, WI), preceding the ATG start codon. The recombinant vectors pAS2-1hBH, pAS2-1hBH$_{1-175}$, pAS2-1hBH$_{1-357}$, and pAS2-1hBH$_{194-357}$ were generated as previously described (*8*), and used for the yeast two-hybrid screening and analyses. For expression in CHO-K1 mammalian cells, full-length hBH cDNA was subcloned into a pcDNA3.1Zeo vector (Invitrogen, Carlsbad, CA) as previously described (*10*), thus generating the pcDNA3.1hBHZeo vector. For in vitro transcription and translation of ^{35}S-labeled proteins, we exploited pCite4 vectors (Novagen). The cDNA for L11 in the pGADgh38$_2$ clone, starting at position 13, was excised using *Eco*RI and *Xho*I recognition sites and subcloned into pCite4, thus generating the pCite4L11 vector. The entire cDNA for L29 was excised from the original liver cDNA library clone pACT2-22$_D$ using *Bam*HI and *Xho*I recognition sites, and subcloned into pCite4 vector, thus generating the pCite4L29 vector. The in vitro L11 and L29 products were synthesized according to the manufacturer's directions for the TnT rabbit reticulocyte lysate system (Promega).

pcDNA3.1MycHisC and pcDNA3.1MycHisA mammalian expression vectors (Invitrogen) with the appropriate reading frames of the multiple cloning site were used to subclone L11 and L29 cDNA, respectively, for further transfections and overexpression in CHO-K1 cells. The following primers were designed for amplification of L11 cDNA using pCite4L11 as a template (*Eco*RI and *Hin*dIII recognition sites in italics). The forward primer, 5′-GCG*GAATTC*GGCATG-GGTCAAGGTG-3′, ensured an ATG start codon within a Kozak consensus sequence; the reverse primer, 5′-GG-G*AAGCTT*TTTGCCAGGAA-3′, included a mutated intrinsic stop codon and ensured in-frame downstream fusion to the *MycHis* epitope. We applied a similar approach for subcloning L29 cDNA. The forward primer (*Eco*RI recognition site in italics), 5′-CCG*GAATTC*GCTTAGGGTGCAGACATGGC-3′, utilized an intrinsic translational start within a perfect Kozak consensus sequence (underlined) and had a mutated upstream ATG → AGG sequence so an out-of-frame translational start could be avoided; the reverse primer (*Bam*HI in italics), 5′-GCG*GGATCC*ATATCGACTCT-GAAGCCTTTGT-3′, ensured an in-frame downstream fusion to the *MycHis* epitope.

In Vitro Binding Assays. The expression and purification of GST fusion constructs of hBH, BH$_{170-455}$ or hBH$_{14-82}$, as well as in vitro binding assays were carried out as described previously (*8*). Briefly, ^{35}S-labeled L11 and L29 (3 μL) were incubated with the GST fusion constructs of hBH prebound to glutathione−Sepharose beads (25 μL) in 50 mM NaCl and bovine serum albumin (1 mg/mL) at 4 °C for 1 h. As a control, ^{35}S-labeled proteins were incubated with GST bound to glutathione−Sepharose. The beads were washed four times with 0.1% Nonidet P-40 in PBS, boiled, and loaded onto SDS−PAGE gels. The gels were soaked in fluorographic reagent Amplify (Amersham, Arlington Heights, IL), dried, and exposed to Kodak X-ray film.

Cell Lines and Transfection Procedures. Untransfected Chinese hamster ovary cells (CHO-K1) and a stable hBH-expressing CHO-hBH cell line (*10*) were maintained in Ham F-12 medium, supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 10 μg/mL streptomycin sulfate, and 10% v/v heat-inactivated fetal bovine serum in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. Stable transfection of pcDNAL11MycHis or pcDNAL29MycHis was carried out in CHO-hBH cells (6 μg of DNA per 25 cm^2 growth area) with SuperFect (Qiagen, Carlsbad, CA) according to the manufacturer's protocol. CHO-hBHL11 and CHO-hBHL29 cell lines were maintained in 500 μg/mL Zeocin (Invitrogen) and 500 μg/mL Geneticin (Gibco Life Technologies).

Immunocytochemistry. CHO-hBHL11 and CHO-hBHL29 cell lines were split onto four-well Permanox chamber slides (Nalge Nunc International, Naperville, IL) and at 50−80% confluence were fixed in 4% paraformaldehyde. After three washings with PBS, cells were permeabilized with 0.2% Triton X-100 for 5 min and nonspecific binding of antibodies was prevented with a 30 min incubation at room temperature with a blocking solution containing 2% bovine serum albumin/0.5% normal goat serum in PBS. Cells were then incubated with the primary antibodies: mouse monoclonal anti-Myc antibody (Calbiochem, Cambridge, MA) diluted 1:100 and a rabbit polyclonal anti-hBH antibody (generously provided by S. A. Johnston, University of Texas, Dallas, TX) diluted 1:250. We used Alexa546 goat anti-rabbit (red) and Alexa488 goat anti-mouse (green) secondary antibodies (Molecular Probes, Eugene, OR). Slides were washed and mounted in Mowiol (Calbiochem, San Diego, CA). Images were collected on three-dimensional data sets using a Photometrics cooled CCD camera and Zeiss Axiovert microscope and deconvolved with ONCOR Image software at 0.2 μm vertical separation.

Subcellular Fractionation. CHO-K1, CHO-hBH, CHO-hBHL11, and CHO-hBHL29 cells were grown to 90% confluency on T150 tissue culture dishes. Membranes were isolated by differential centrifugation. Briefly, cells were detached from the dishes by incubation with 10 mM EDTA in PBS for 20 min. Cells were pelleted at 1000*g* for 10 min and resuspended in hypotonic buffer [10 mM Tris (pH 7.4), 0.2 mM $MgCl_2$, 5 mM KCl, and protease inhibitors (1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 0.1 μg/mL pepstatin)] to swell. After a 20 min incubation on ice, the medium was adjusted to isotonic conditions by addition of sucrose (0.25 M final concentration) and the cells were disrupted with 25−30 passes in a Dounce homogenizer with a tight pestle until >90% of the cells were broken as determined by Trypan Blue dye exclusion. Subcellular

fractionation was accomplished by sequential differential centrifugation to yield pellets of nondisrupted cells and cellular nuclei (1000*g* for 10 min) and mitochondria (10000*g* for 20 min). The postmitochondrial supernatant was centrifuged at 100000*g* for 90 min using a 75 Ti rotor in a Beckman XL-70 ultracentrifuge to yield ribosomal pellet and cytosolic supernatant fractions. Each pellet was washed once by resuspension in isotonic buffer, centrifuged under the respective condition, and finally resuspended in the same buffer. Assays for BH activity and organelle marker enzymes were performed on freshly prepared subcellular fractions as described below. hBH, L11, and L29 were also visualized by immunoblotting proportional aliquots from each subcellular fraction with anti-T7 and anti-Myc monoclonal antibodies, respectively.

In Vitro Binding of His-hBH to CHO-K1 Ribosomes. In vitro binding of His-hBH to CHO-K1 ribosomes was evaluated as described previously with a slight modification (*14*). Briefly, the postmitochondrial supernatant fraction obtained by differential centrifugation of homogenates of 2 $\times$ 10^6 CHO-K1 cells was separated into 600 μL aliquots. To each aliquot we added 20 μL of His-hBH to yield the following final concentrations: 4, 1, 0.25, 0.0625, and 0.0125 μg/mL. After a 10 min incubation at room temperature, the tubes were centrifuged at 230000*g* for 30 min with a 100.3 rotor in a TL 100 ultracentrifuge. Ribosomal pellets were resuspended in SDS sample buffer, analyzed by SDS−PAGE, and immunoblotted with anti-Xpress monoclonal antibody (Invitrogen) diluted 1:5000.

Bleomycin Hydrolase and Other Enzymatic Assays. BH activity was examined with aliquots from each cell fraction (approximately 100 μg of total protein) by our previously described HPLC method that separates bleomycin A_2 from its inactive metabolite deamidobleomycin A_2 (dA_2) (*8*, *15*). Assays for succinate dehydrogenase for mitochondria and NADPH cytochrome *c* reductase for endoplasmic reticulum were performed as described previously (*16*). Assays for lactate dehydrogenase for the cytosolic fraction and 5′-nucleotidase for plasma membranes were performed using diagnostic kits obtained from Sigma.

Analytical Procedures. Protein concentrations in cell lysates were assessed by the Bradford assay (Bio-Rad, Hercules, CA). SDS−PAGE was performed on 12% gels (Novex, San Diego, CA), and proteins were transferred to nitrocellulose membrane, probed with antibodies, and detected by Renaissance chemiluminescence reagent (NEN Life Science Products, Inc., Boston, MA). Affinity-purified His-tagged hBH containing an Xpress epitope (His-hBH) was prepared as described previously (*8*).

RESULTS

Yeast Two-Hybrid System. To identify heterologous partners of hBH, we cloned a cDNA sequence encoding the 455-amino acid full-length hBH into the GAL4-based two-hybrid vector pAS2-1 (pAS2-1hBH) and cotransformed yeast Y190 strain with pAS2-1hBH and the HeLa cDNA library cloned into the activating domain vector pGADgh as previously described (*8*). Approximately 10^6 clones were screened, and three were found to be true positives after mating with pAS2-1hBH and control pAS2-1 vectors. Two of them, a truncated form of hBH and ubiquitin-conjugating enzyme 9, have already been reported (*8*, *10*), and the third was a ribosomal protein, human ribosomal protein L11. We also screened approximately 10^7 clones from a human liver library cloned into the activating domain vector pACT2, and among several true positives, a second ribosomal protein, namely, human L29, was identified. The β-galactosidase assay was positive when plasmids encoding hBH and L11 or hBH and L29 were coexpressed (Figure 1A,C). The specificity of interaction between hBH and L11 or hBH and L29 was confirmed by the lack of detectable β-galactosidase activity when the hBH was replaced by three unrelated proteins or BD only (Figure 1A,C). To define further the amino acid sequences that are important for the interaction of hBH and L11, we constructed various N- and C-terminal truncations of hBH (Figure 2B). Yeast were cotransformed with plasmids expressing hBH

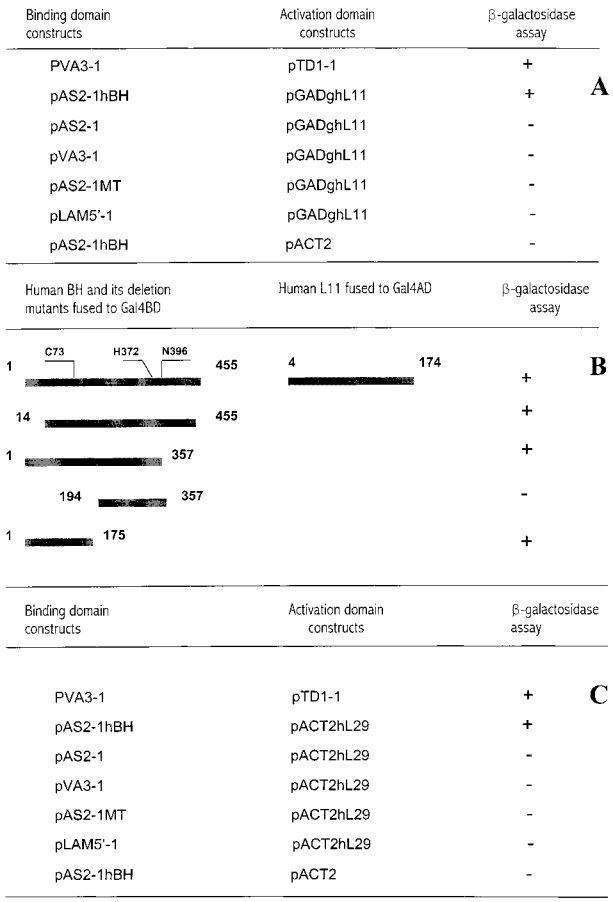

FIGURE 1: hBH interacts with L11 and L29 in a two-hybrid system. (A) Specificity of hBH−L11 interaction in the yeast two-hybrid assay. Yeast 190 strain cells were transformed with plasmid combinations of hBH (pAS2−1hBH) or unrelated proteins: murine p53 (pVA3-1), metallothionein (pAS2-1MT), lamin (pLAM5-1) fused to the Gal4BD (binding domain) or of GAL4BD alone in the pAS2-1 plasmid, and human L11 fused to the Gal4AD (activation domain) in the pGADgh plasmid. (B) hBH_{1-175} is the smallest region of hBH interacting with L11. Yeast 190 strain cells were transformed with different combinations of hBH deletion mutants fused to the Gal4BD and human L11 fused to the Gal4AD in the pGADgh plasmid. (C) Interaction between hBH and L29 in a two-hybrid system is specific. Yeast 190 strain cells were transformed with plasmid combinations of human BH or unrelated proteins fused to the Gal4BD in pAS2-1 and human L29 fused to the Gal4AD in pACT2 plasmids. The interaction between p53 (pVA3-1) and large T antigen (pTD1-1) was used as a positive control. β-Galactosidase activity was monitored by using the qualitative filter assay and scored + if blue color developed and − if no color appeared.

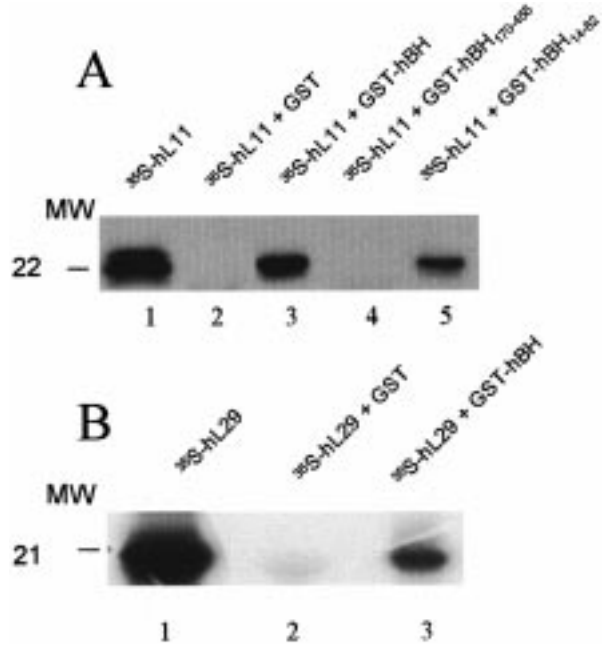

FIGURE 2: In vitro binding assays of ^{35}S-labeled L11 and ^{35}S-labeled L29 and GST−hBH fusion proteins immobilized on glutathione−agarose. (A) L11 binds to hBH in vitro. ^{35}S-labeled L11 (lane 1) was precipitated with GST-tagged hBH and hBH_{14-82} (lanes 3 and 5), but not with GST alone or GST−$hBH_{170-455}$ (lanes 2 and 4). (B) L29 binds to hBH in vitro. ^{35}S-labeled L29 (lane 1) was precipitated with GST-tagged hBH (lane 3), but not with GST alone (lane 2). Precipitated proteins were separated on 12% SDS−PAGE gels, dried, and exposed to X-ray film.

deletion mutants and L11 and assayed for β-galactosidase activity. The smallest deletion of hBH that interacted with L11 was hBH_{1-175}, which contains only one of the active sites of hBH, namely, Cys^{73}. The other two-thirds of the hBH molecule, which contained two other active sites, His^{372} and Asp^{392}, was dispensable for binding with L11.

hBH Interacts with L11 and L29 in Vitro. To verify the interaction between hBH and L11, we performed in vitro binding assays using full-length hBH as well as two N- and C-terminal hBH deletion mutants expressed as N-terminal GST fusion proteins in *Escherichia coli*. GST fusion proteins were first immobilized on glutathione−Sepharose beads. The beads were then incubated with in vitro transcribed and translated ^{35}S-labeled protein corresponding to L11. As a negative control, ^{35}S-labeled proteins were incubated with GST protein alone bound to glutathione−Sepharose beads. As is evident in Figure 2A, ^{35}S-labeled L11 bound specifically to GST−hBH and GST−hBH_{14-82} but did not bind to GST−$hBH_{170-455}$ and GST. The same in vitro binding assay was applied for L29, without hBH deletion constructs. As is evident in Figure 2B, ^{35}S-labeled L29 also interacted with GST−hBH but not with GST (lanes 3 and 2, respectively).

hBH Colocalizes with Ribosomal Proteins L11 and L29. To investigate localization of hBH and ribosomal proteins, we established CHO-hBHL11 and CHO-hBHL29 cell lines. hBH was tagged with the T7 epitope, and L11 and L29 were fused to the Myc epitope. CHO-hBHL11 and CHO-hBHL29 cell lines were analyzed using indirect immunofluorescence and deconvoluting microscopy. hBH (Figures 3B and 4B) is mostly cytosolic with intense perinuclear staining, though concentrated on the outer site of the nuclear membrane. No plasma membrane staining was observed, although there

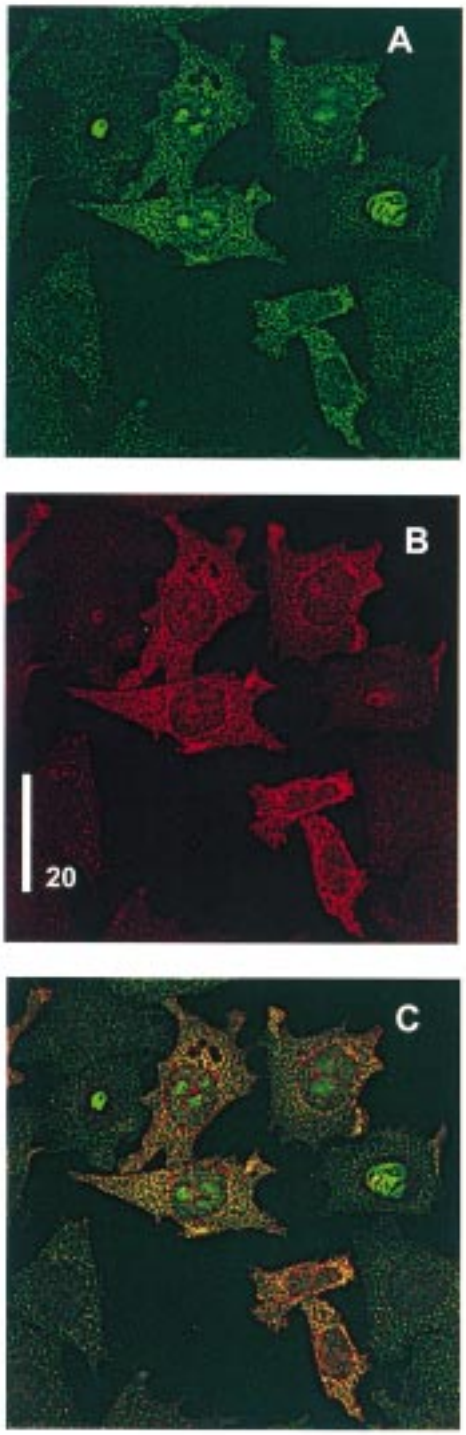

FIGURE 3: Colocalization of hBH with L11 in CHO-hBHL11 cells. CHO cells were stably transfected with plasmids encoding hBH and L11Myc. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Myc-tagged L11 was immunostained with anti-Myc mAb and Alexa488-conjugated (green) goat anti-mouse secondary antibodies (A). hBH was visualized with polyclonal anti-hBH antiserum and Alexa546-conjugated (red) goat anti-rabbit secondary antibodies (B). Overlays are shown in panel C. Orange indicates the region of overlap. Images were collected using Zeiss Axiovert and deconvoluted with ONCOR Image software.

appeared to be faint staining of the nucleoplasm heterochromatin, as judged by the exclusion of the nucleoli. The localization profile of hBH indicated the enzyme was

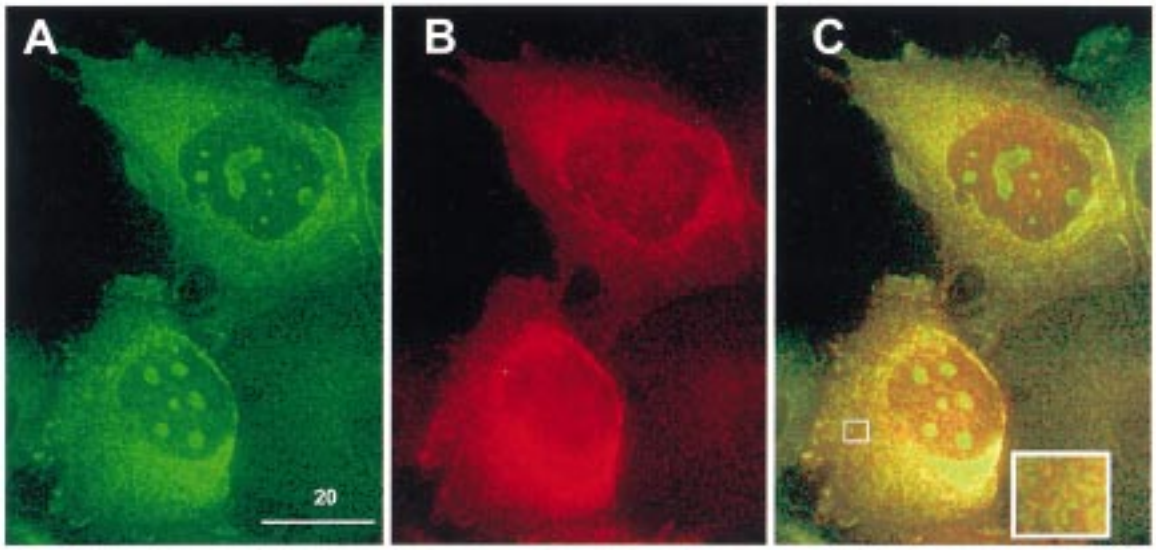

FIGURE 4: Colocalization of hBH with L29 in CHO-hBHL29 cells. CHO cells were stably transfected with plasmids encoding hBH and L29Myc. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Myc-tagged L29 was immunostained with anti-Myc mAb and Alexa488-conjugated (green) goat anti-mouse secondary antibodies (A). hBH was visualized with polyclonal anti-hBH antiserum and Alexa546-conjugated (red) goat anti-rabbit secondary antibodies (B). Overlays are shown in panel C. The inset shows a region of overlap. Orange indicates the region of overlap. Images were collected using Zeiss Axiovert and deconvoluted with ONCOR Image software.

Table 1: Distribution of Bleomycin Hydrolase and the Subcellular Compartment Marker Enzymes after Cell Fractionation of CHO Cells[a]

fraction	protein (mg)	bleomycin hydrolase (%)	succinate dehydrogenase (%)	LDH (%)	NADPH cytochrome *c* reductase (%)	5′-nucleotidase (%)
mitochondrial (10000*g* for 20 min)	0.68 ± 0.1	5.6 ± 1.8	91.6 ± 4.2	0.7 ± 0.5	4.7 ± 3.5	54.4 ± 9.2
ribosomal (100000*g* for 90 min)	0.78 ± 0.1	21.0 ± 3.7	7.9 ± 4.3	2.8 ± 0.3	39.2 ± 5.3	31.1 ± 7.5
cytosolic	3.4 ± 0.4	71.9 ± 4.5	0.49 ± 0.6	96.7 ± 0.9	56.0 ± 8.3	20.5 ± 7.1

[a] CHO-hBH, CHO-hBHL11, and CHO-hBHL29 cells were subjected to isotonic lysis and differential centrifugation as described in Experimental Procedures. Each subcellular fraction was analyzed for the distribution of BH activity and marker enzymes specific for various organelles: succinate dehydrogenase (mitochondria), lactate dehydrogenase (cytosol), NADPH cytochrome *c* reductase (endoplasmic reticulum), and 5′-nucleotidase (plasma membrane). The data are from nine independent experiments, three from each of the indicated cell lines. The sum of specific activities (millimoles per minute per milligram) in mitochondrial, ribosomal, and cytosolic fractions is arbitrarily defined as 100%. Values given for marker enzyme in each fraction represent the percentage of the total activity ± the standard error.

cytoplasmic and might be associated with the membranes of the endoplasmic reticulum. L11 and L29 (Figures 3A and 4A) also exhibited cytoplasmic labeling, but there was also intense nucleolar staining that corresponds with their predicted cellular localization. The overlays (Figures 3C and 4C) showed intensive yellow/orange colors in the cytosol, indicating colocalization of the two proteins. No evidence of colocalization was seen in the nuclei of stained cells.

Subcellular Distribution of hBH and Ribosomal Proteins. Subcellular fractionation of CHO-hBH, CHO-hBHL11, and CHO-hBHL29 cell extracts was attempted to determine if hBH fractionated with ribosomal proteins. Coexpression of either L11 or L29 with hBH did not significantly alter the distribution of BH, the ribosomal proteins, or compartment marker enzyme among cell lines, and therefore, the results from all three cell lines were pooled (Table 1). Cell extracts were prepared in isotonic buffer containing 0.25 M sucrose and separated into nuclear (1000*g* for 10 min), mitochondial (10000*g* for 20 min), ribosomal (100000*g* for 90 min), and cytosolic supernatant (100000*g*) fractions by differential centrifugation. Aliquots of each fraction, corresponding to 50 μg of total protein, were used for Western immunoblotting. As shown in Figures 5A and 6A, hBH was found mostly

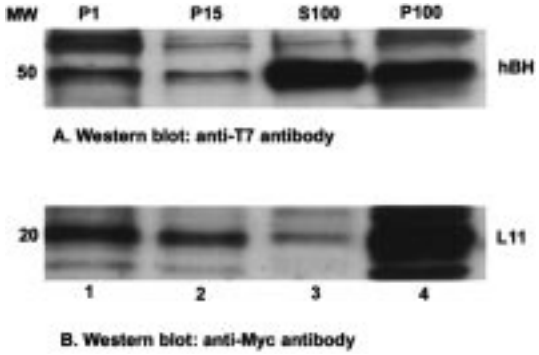

FIGURE 5: Immunoblotting of hBH and L11 in subcellular fractions from CHO-hBHL11 cells. Subcellular fractions were prepared from CHO-hBHL11 cells and analyzed by SDS−PAGE and Western blotting as described in Experimental Procedures. Approximately 50 μg of total protein was added per lane: lane 1 (P1), 1000*g*, 10 min pellet; lane 2 (P10), 10000*g*, 20 min pellet; lane 3 (S100), 100000*g*, 90 min supernatant; and lane 4 (P100), 100000*g*, 90 min pellet. (A) The Western blot was probed with anti-T7 monoclonal antibodies. (B) The Western blot was probed with anti-Myc antibodies. The migration positions of hBH and L11 are denoted.

in cytosolic supernatant (S100), although abundant hBH was found in the 100000*g*, 90 min pellet (P100 in Figures 5 and

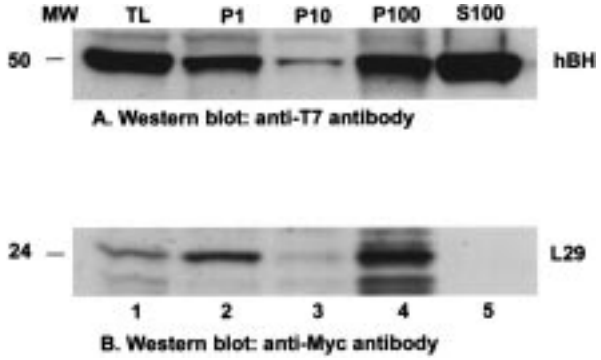

FIGURE 6: Immunoblotting of hBH and L29 in subcellular fractions from CHO-hBHL29 cells. Subcellular fractions were prepared from CHO-hBHL29 cells and analyzed by SDS−PAGE and Western blotting as described in Experimental Procedures. Approximately 50 μg of total protein was added per lane: lane 1 (TL), total CHO-hBHL29 lysate; lane 2 (P1), 1000*g*, 10 min pellet; lane 3 (P10), 10000*g*, 20 min pellet; lane 4 (P100), 100000*g*, 90 min pellet; and lane 5 (S100), 100000*g*, 90 min supernatant. (A) The Western blot was probed with anti-T7 monoclonal antibodies. (B) The Western blot was probed with anti-Myc antibodies. The migration positions of hBH and L29 are denoted.

6) with a relative band intensity that was $^1/_3$ of that of the S100 band. hBH, L11, and L29 were also detected in other particulate fractions, 1000*g*, 10 min pellet (P1 in Figures 5 and 6), probably because of the presence unbroken cells, and a small amount was visible in 10000*g*, 20 min pellet (P10 in Figures 5 and 6). L29 was depleted from the S100 fraction (Figure 6, lane 5), but a faint band corresponding to L11 was detected in the 100000*g* soluble fraction (Figure 5, lane 3). As shown previously (*17*, *18*), L29/HIP migrated as a 24 kDa protein rather than at 17.7 kDa, probably due to the highly basic character of L29/HIP. Each subcellular fraction was assayed for BH and organelle marker enzyme activities (succinate dehydrogenase, mitochondria), NADPH cytochrome *c* reductase (endoplasmic reticulum), 5′-nucleotidase (plasma membrane), and lactate dehydrogenase (cytosol) (Table 1). The enriched fraction for most of the BH activity (60−80%) was the cytosol fraction, but the 100000*g*, 90 min particulate fraction had a considerable relative amount of hBH (21.0 ± 3.7%). The high BH activity in this fraction cannot be explained by cytoplasmic contamination. Therefore, we concluded that a significant fraction of the hBH could be associated with ribosomes.

hBH Binds to the Microsomal Fraction in Vitro. Binding of hBH to microsomes, which contain ribosomes, was examined in vitro. Serial dilutions of affinity-purified recombinant hBH (His-hBH) were incubated with CHO-K1 postmitochondrial supernatant, and the amount of His-hBH bound to the microsomal pellet was determined by anti-Xpress immunoblotting after centrifugation. As is evident in Figure 7, the binding of His-hBH to microsomes, which contain ribosomes, was concentration-dependent.

DISCUSSION

BH is an unusual multifunctional cysteine proteinase. BH is expressed in most tissues and has been well-preserved during the course of evolution, insinuating an important but still ill-defined cellular role. Recently, using a yeast two-hybrid system, we found hBH interacted with the human homologue of UBC9 (*10*). Now we report binding and colocalization of hBH with ribosomal proteins.

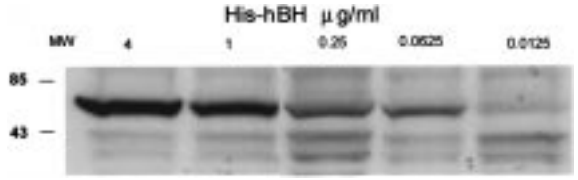

FIGURE 7: Binding of recombinant His-hBH to CHO-K1 ribosomes. Different concentrations (micrograms per milliliter) of affinity-purified recombinant His-hBH and equal-sized aliquots of the postmitochondrial supernatant (10000*g* for 20 min) from CHO-K1 cells were incubated for 10 min at room temperature and centrifuged at 230000*g* for 30 min at 4 °C. The resulting ribosomal pellet was analyzed by SDS−PAGE and immunoblotted with anti-Xpress monoclonal antibody. The migration position of hBH is denoted at the right and that of the molecular mass marker protein (in kilodaltons) at the left.

BH was previously believed to be a cytosolic protein. yBH was found in the cytoplasm of yeast cells (*5*). Recently, using indirect immunofluorescence, we showed epitope-tagged hBH is cytosolic with intensive perinuclear staining (*10*). There have been some previous indications, however, that BH may be associated with cellular membranes. Magdolen et al. (*19*) copurified yBH with Gce1p, cAMP-binding ectoprotein anchored to the plasma membrane by virtue of a glycosyl−phosphatidylinositol anchor. Kambouris et al. (*20*) isolated yBH as an amphitropic protein occurring both in the cytoplasm and bound to the plasma membrane on the basis of binding to phospholipids in a calcium-dependent manner. Thus, we have used three converging methodologies to test the hypothesis that hBH binds to membranous structures. We show part of hBH is fractionated in the high-speed particulate fraction together with two ribosomal proteins and that the microsomal fraction was considerably enriched with bleomycin hydrolyzing activity (~20%). We also demonstrate in vitro binding of hBH to CHO-K1 microsomes, which contain ribosomal proteins. Binding of BH to ribosomal proteins may assist its attachment to ribosomes and membranes of the endoplasmic reticulum, because ribosomes cycle between the membrane-bound and unbound state (*21*). Thus, BH may also interact with the cytoplasmic ribosomes. Because BH does not have any obvious hydrophobic or plausible transmembrane domain, association with cellular membranes might be indirect through another protein partner. Candidates for such a role are the now reported L11 and L29 ribosomal proteins. Ribosomal proteins L11 and L29 are associated with the large ribosomal subunit. Because L11 interacted with the N-terminus of BH (*17*, *18*) which is critical for oligomerization and catalytic activity, it is interesting to speculate that L11 might block oligomerization and enzyme activity. We found, however, L11 did not affect the ability of BH to metabolize bleomycin (data not shown). Additional studies will be required to answer whether L11 affects hBH oligomerization. The human analogue of L29 was unexpectedly described as a heparin/heparan interacting protein (HIP) (*17*, *18*). Using immunostaining of fixed, nonpermeabilized cells, at least some L29 has been localized on the cell surface of transfected and human uterine epithelial cells. Our data, however, indicated L29 was mainly intracellular in CHO-K1 cells, which could reflect species differences or the use of a procedure for immunostaining that includes permeabilization of the cells, often obscuring cell surface localization of a

protein. Nonetheless, our results and previously reported data (*18*) indicate the human analogue of L29 fractionated in the ribosomal pellet.

The presence of BH on ribosomes may help inactivation of bleomycin or antibiotics that bind to RNA. Bleomycin is an anticancer drug that binds and cleaves DNA and can also cleave RNA (*22*). There are other cytotoxic proteins that also act on eukaryotic ribosomes. Ricin, for example, irreversibly inactivates the 60S ribosomal subunit (*23*). Therefore, if BH is engaged in detoxification of ribosomal poisons, its presence on the ribosomes would be advantageous. Homologues of hBH from different species may use different ways to associate with ribosome. yBH has been shown to bind RNA in vitro, and this binding was coupled to the bleomycin inactivation, because a yBH mutant deprived of nucleic acid-binding activity was more sensitive to bleomycin than the wild type (*4*, *5*). hBH exhibits more than 40% amino acid identity with yBH, but the loss of the amino acids responsible for nucleic acid binding activity of yBH explains the inability of hBH to bind DNA in yeast two-hybrid system (*8*). Therefore, our finding that hBH binds to ribosomal proteins points to a conserved role in maintaining RNA integrity and suggests we should focus additional attention on these interactions.

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