An Evolutionarily Conserved Cysteine Protease, Human Bleomycin Hydrolase, Binds to the Human Homologue of Ubiquitin-Conjugating Enzyme 9

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ABSTRACT

Bleomycin hydrolase (BH) is a highly conserved cysteine proteinase that deamidates and inactivates the anticancer drug bleomycin. Yeast BH self-assembles to form a homohexameric structure, which resembles a 20 S proteasome and may interact with other proteins. Therefore, we searched for potential human BH (hBH) partners using the yeast two-hybrid system with a HeLa cDNA library and identified the full-length human homologue of yeast ubiquitin-conjugating enzyme 9 (UBC9). Cotransformation assays using hBH deletion mutants revealed that the carboxyl terminus of hBH (amino acids 356–455), which contains two of the three essential catalytic amino acids, was not critical for protein binding in the yeast two-hybrid environment. In vitro translated human UBC9 was precipitated by glutathione S-transferase-hBH fusion protein but not by glutathione S-transferase. Efficient in vitro binding occurred in the absence of the first 24 amino acids of UBC9 and the catalytic Cys93 of UBC9. We confirmed that hBH and UBC9 interacted in vivo by affinity copurification of proteins overexpressed in mammalian cells. Using immunocytochemical analysis, hBH was colocalized with UBC9. Coexpression of hBH and UBC9 in mammalian cells did not markedly alter the bleomycin-hydrolyzing activity of hBH or apparent small ubiquitin-related modifier 1 addition. This is the first reported heteromeric interaction with hBH, and it suggests a role for hBH in intracellular protein processing and degradation.

BH is a 455-amino acid cysteine proteinase that degrades the anticancer drug bleomycin and thus confers bleomycin resistance. BH is conserved among eukaryotes, with >40% identity between yBH and hBH (Berti and Storer, 1995). BH orthologues have also been identified in bacteria (Mistou et al., 1994). The aminopeptidase activity of BH is well established and presumably is responsible for bleomycin deamidation. BH may have other roles superseding that of an aminopeptidase. For example, the yeast gene for BH (BLH1) was identified (Enenkel and Wolf, 1993) as a gene encoding a protein that suppresses the in vitro phenotype of the pre3–2 mutant yeast strain, which is defective in one of the catalytic subunits of the yeast proteasome and is devoid of Cbz-Leu-Leu-Glu-β-naphthylamide-hydrolyzing activity. Magdolen et al. (1993) copurified yBH with Gee1p, which is a cAMP-binding ectoprotein anchored to the plasma membrane by glycosyl-phosphatidylinositol. yBH binds DNA and has an unusual regulatory function as a member of the galactose regulon in yeast. This regulatory activity seems to be independent of both the protease and DNA-binding activities and could reflect interactions with other protein partners (Zheng et al., 1997). The crystal structure of yBH reveals a hexameric structure with a narrow axial channel leading to a cavity containing the active sites, resembling the organization of the proteasome (Joshua-Tor et al., 1995). We recently found that hBH has intrinsic endopeptidase activity (Koldamova et al., 1998), and others have characterized the unusual autocarboxypeptidase and peptide ligase activities of yBH (Zheng et al., 1998).

The hBH gene is widely expressed by normal tissues (Bromme et al., 1996), which is consistent with a proposed role for this proteolytic enzyme in normal protein catabolism (Ferrando et al., 1996). hBH expression is transcriptionally regulated and, like many housekeeping genes, the 5′-flanking region of the hBH gene lacks consensus transcriptional

ABBREVIATIONS: BH, bleomycin hydrolase; yBH, yeast bleomycin hydrolase; hBH, human bleomycin hydrolase; PBS, phosphate-buffered saline; UBC9, ubiquitin-conjugating enzyme 9; hUBC9, human ubiquitin-conjugating enzyme 9; BHYD, bleomycin hydrolase unique domain; SUMO-1, small ubiquitin-related modifier 1; dA, deamidobleomycin A2; CHO, Chinese hamster ovary; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEK, human embryonic kidney; ΔUBC9, truncated ubiquitin-conjugating enzyme 9; ΔBH, truncated bleomycin hydrolase; GST, glutathione S-transferase; GAP, GTPase-activating protein; Nt-NTA, Nt2+ nitrilotriacetic resin.
sequences, such as TATA or CCAAT boxes (Ferrando et al., 1997). There is no evidence, however, that hBH interacts with other proteins.

Like yBH, hBH forms dimers that permit the formation of homotetrameric and homohexameric structures. Because of the proposed structural similarities between BH and the 20 S proteasome (Joshua-Tor et al., 1995), we hypothesized that hBH is also engaged in heteromeric interactions. We now report the first heterologous hBH-binding protein, the human homologue of UBC9.

**Materials and Methods**

**Plasmids and yeast two-hybrid screening.** Yeast two-hybrid screening of a HeLa cDNA library and analysis were performed as previously described (Koldamova et al., 1998), using 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, gal4Δ, gal80Δ, cyh2, 5′-GCGAATTCAGTGGCTACTCGGCTGTTG-3′ (forward) and 5′-GGAAATTCGTCGTCGAGCTCATGAG-3′ (reverse). The reaction mixtures (50-μl final volume) consisted of 10 ng of double-stranded DNA vector (either pcite4UBC9 or pcite4UBC9C) 125 ng of each oligonucleotide primer, nucleotide triphosphates, buffer, and Pyrococcus furiosus DNA polymerase, according to the manufacturer’s recommendations. The reaction was cycled in a PTC-200 thermal cycler (MJ Research, Watertown, MA) with steps of 95° for 30 sec, 55° for 1 min, and 68° for 12 min, which were repeated 12 times. After temperature cycling, the reaction tubes were cooled on ice for 2 min and incubated for 2 hr at 37° with 10 units of DpnI restriction enzyme, to digest the parental, unnmutated, supercoiled, double-stranded DNA. We used 2 μl of DpnI-treated DNA to transform MaxEfficiency DH5α competent cells (Gibco BRL, Grand Island, NY). The in-frame position of all cDNA inserts was confirmed by dye terminator labeling and sequencing, using an ABI Prism 373 DNA sequencer (University of Pittsburgh Research Facility).

**In vitro binding assays.** GST fusion constructs of hBH, ΔBH (hBH14–455), or hBH14–357 were expressed in Escherichia coli DH5α and affinity-purified on glutathione-Sepharose (Pharmacia) as described previously (Koldamova et al., 1998). Briefly, 35S-labeled UBC9, ΔUBC9, or UBC9Trp93 (3 μl) was incubated at 4° with 1 μg of GST-fusion constructs of hBH bound to glutathione-Sepharose. The beads were washed four times with 0.1% Nonidet P-40 in PBS, boiled, and loaded on SDS-polyacrylamide gels. The gels were soaked in fluorescent reagent (Amplify; Amersham, Arlington Heights, IL), dried, and exposed to Kodak X-ray film.

**Cell lines and transfection procedures.** CHO cells were cultured in Ham F-12 medium and HEK293 cells were cultured in Dulbecco’s modified Eagle medium. Media were supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 10 μg/ml streptomycin sulfate, and 10% (v/v) heat-inactivated fetal bovine serum, and the cell cultures were maintained at 37° in a humidified atmosphere of 95% air/5% CO2. Lipofectamine (Gibco BRL) was used for transfection, according to the manufacturer’s protocol, with corresponding recombinant mammalian expression vectors. Established cell lines were maintained using 400–500 μg/ml concentrations of Geneticin (G418).
(Gibco BRL) or Zeocin (Invitrogen). Transient expression of hBH and UBC9 was achieved in HEK293 and CHO cells using 6–12 μg of DNA/25-cm² growth area.

Preparation of cell lysates, affinity purification on Ni-NTA-agarose, SDS-PAGE, and Western blotting. Cell lysates from CHO and HEK293 cells were prepared as follows. Approximately 3 × 10⁶ cells were incubated for 10 min on ice with 0.4 ml of RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotonin). Cells were sonicated, and lysates were cleared of nuclei and debris by centrifugation at 14,000 × g for 40 min. The supernatants were saved and used for Western blotting, affinity purification on Ni-NTA-agarose, and BH activity assays. Protein concentrations in cell lysates were determined by using the Bradford assay (Bio-Rad, Hercules, CA).

Affinity purification on Ni-NTA-agarose was as described (Koldamova et al., 1998). Briefly, for copurification experiments, cell lysates were prepared from HEK293 cells that had been mock-transfected or transiently transfected with hBH, histidine-tagged UBC9, or hBH plus histidine-tagged UBC9. To isolate histidine-tagged proteins, we incubated equal amounts of total protein from each sample (up to 200 μg) with Ni-NTA-agarose (50% slurry), equilibrated with a buffer (20 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, 20 mM imidazole, 100 mM KCl, 10% glycerol, 0.1% Nonidet P-40), for 1 or 8 hr at 4°C. The bound proteins were washed five times and eluted with SDS sample buffer (0.25% Tris, pH 6.8, 2.5% SDS, 0.05% bromphenol blue, 10% glycerol, 2.5% β-mercaptoethanol) and were loaded onto SDS-polyacrylamide gels. Proteins were separated, after boiling, by 4–20% gradient SDS-PAGE. We performed Western immunoblotting with AntiXpress (diluted 1/5,000; Invitrogen) or anti-T7 (diluted 1/10,000; Novagen) antibodies, followed by horse-radish peroxidase-conjugated polyclonal goat anti-mouse IgG (diluted 1/3,000). Signals were detected using a chemiluminescence detection assay (NEN, Boston, MA) and a 3-min exposure to X-ray film.

Subcellular fractionation. Subcellular fractionation of CHO cells and CHO cells transfected with pcDNA3.1hBHZeol was performed as described previously (Kamitani et al., 1997). Briefly, to prepare S-100 and P-100 fractions, 3 × 10⁶ cells were washed with PBS, resuspended in 2 ml of hypotonic lysis buffer (5 mM Tris-HCl, pH 7.4, 2.5 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, with protease inhibitors), and incubated on ice for 15 min (to swell the cells). The cell suspension was homogenized by using a Dounce homogenizer. The homogenate was centrifuged at 1000 × g for 3 min to remove nuclei and undisrupted cells. The supernatant was centrifuged at 100,000 × g for 1 hr. The pellet was solubilized with 200 μl of 2% SDS solution and used as the P-100 fraction. The supernatant was concentrated with Centricon-10 filters (Amicon, Beverly, MA) to a final volume of 100 μl, mixed with 100 μl of 4% SDS solution, and used as the S-100 fraction. For preparation of a nuclear fraction, 3 × 10⁶ cells were washed with PBS, resuspended in 2 ml of hypotonic lysis buffer, and incubated on ice for 15 min, followed by Dounce homogenization. The homogenate was overlaid on 5 ml of lysis buffer containing 0.5 M sucrose and was centrifuged at 3000 × g for 10 min. The pellet was solubilized with 200 μl of 2% SDS solution and used as the nuclear fraction. An aliquot of each fraction was loaded on a 4–20% gradient gel, transferred to a nitrocellulose membrane, and probed with monoclonal anti-SUMO-1 antibody (Zymed, South San Francisco, CA) at a concentration of 0.5 μg/ml.

Immunocytochemical analysis. CHO cells were split onto four-well Permanox chamber slides (Nalge Nunc International, Naperville, IL) and at 50–80% confluence were transiently transfected using SuperFect (Qiagen) and 1.5 μg of DNA of the appropriate mammalian expression vector (either pcDNA3.1UBC9Mychis or pcDNA3.1hBHZeol) for each chamber. Twenty-four hours later, the cells were washed with PBS and fixed in PBS containing 4% paraformaldehyde. After three washes with PBS, cells were permeabilized with 0.2% Triton X-100 for 5 min, and nonspecific binding of antibodies was blocked with blocking solution (2% bovine serum albumin and 0.5% normal goat serum in PBS) for 30 min at room temperature. Cells were then incubated with the primary antibodies, namely rabbit polyclonal anti-Myc (Upstate Biotechnology, Lake Placid, NY) or monoclonal anti-T7 (Novagen), at the appropriate dilutions (1/3000 and 1/1000, respectively) in blocking solution. After 1 hr, the cells were washed with 1% Triton X-100 in PBS and incubated for 60 min at room temperature with secondary antibodies (Cy3-conjugated goat anti-rabbit antibody at a 1/2000 dilution and fluorescein isothiocyanate-conjugated goat anti-mouse antibody at a 1/400 dilution) (Cy3 from Research Organics, Cleveland, OH). Slides were washed, mounted in Mowiol (Calbiochem, San Diego, CA), and analyzed using conventional (Nikon Microphot) and confocal (Molecular Dynamics) microscopy.

BH assay. The metabolism of bleomycin was assessed using our previously described high performance liquid chromatographic method, which separates bleomycin A₂ from its inactive metabolite daA₂ (Sebit et al., 1987). Briefly, cell lysates (6 μg/ml total protein) prepared from CHO and HEK293 cells that had been transiently transfected with hBH or UBC9 or cotransfected with hBH and UBC9 were incubated with 70 μl bleomycin A₂ (Bristol Myers Squibb, Wallingford, CT, or Nippon Kayaku, Tokyo, Japan), in 50 μl of reaction buffer (20 mM Tris, pH 7.5), at 37° for 2 hr. The reaction was stopped by addition of 40 μl of methanol and 10 μl of 7.5 mM CuSO₄, the mixture was centrifuged, and the resulting supernatant fractions were injected onto a C₁₈ reverse-phase high performance liquid chromatography column (3.9 mm × 150 mm, 5-μm particle size; Waters Chromatography, Milford, MA). Bleomycin A₂ and daA₂ were eluted at 1 ml/min with a solution of 17% methanol, 7.2% acetonitrile, 0.8% acetic acid, 2 mM heptanesulfonic acid, and 25 mM triethylamine (pH 5.5) and were detected using absorbance measurements at 292 nm. The bleomycin-hydrolyzing activity of hBH was defined as the percentage of the total amount of bleomycin A₂ converted into daA₂ during the incubation period.

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 strain Y190 with pAS2–1hBH and the HeLa cDNA library sequence cloned into the activating domain vector pGADgh, as previously described (Koldamova et al., 1998). Approximately 10⁹ clones were screened; among the 98 His⁺/lacZ⁺ clones, 4 were found to be true positive after mating with pAS-1hBH and control pAS-1 vectors. One, an amino-terminally truncated form of hBH without the first 13 amino acids (ABH), has already been reported (Koldamova et al., 1998); two others were ribosomal proteins and are still being evaluated. The fourth (original library clone pGADgh113 1), which we now present, contains the full-length coding sequence of hUBC9 flanked by 5’- and 3’-untranslated regions (Fig. 1). Intense β-galactosidase activity was seen when plasmids encoding hBH and UBC9 were coexpressed (Fig. 2A). The specificity of the UBC9 interaction with hBH was confirmed by the lack of detectable β-galactosidase activity when the UBC9-related construct or the hBH-related construct was replaced with a vector-only construct or constructs encoding three irrelevant proteins (Fig. 2A). To further define the amino acid sequences important for interaction of hBH and hUBC9, we constructed various amino- and carboxyl-terminal truncations of hBH (Fig. 2B). Yeast were cotransformed with plasmids expressing different deletion mutants and were assayed for β-galactosidase activity. The smallest deletion mutant of hBH that interacted with hUBC9 was hBH₁₋₃₅₇, which contains only one of the active sites of hBH,
namely Cys73. We also performed quantitative analysis using liquid β-galactosidase assays, which fully confirmed the qualitative data (data not shown). Therefore, hBH did not require all three active sites for interaction with UBC9 and, in particular, did not require the unique and highly conserved BHYD, which is essential for aminopeptidase and bleomycin-hydrolyzing activities (Koldamova et al., 1998).

**hBH/hUBC9 interaction in vitro.** To verify the interaction between hBH and UBC9, we performed in vitro binding assays using full-length hBH as well as two amino- and carboxyl-terminal hBH deletion mutants expressed as GST-fusion proteins in E. coli. GST-fusion proteins were first immobilized on glutathione-Sepharose beads. The beads were then incubated with in vitro transcribed and translated 35S-labeled proteins corresponding to the full-length coding sequence of UBC9, an amino-terminal deletion mutant without the first 24 amino acids (DUBC9), or full-length UBC9 in which the active residue Cys93 had been mutated to tryptophan (UBC9Trp93). We generated DUBC9 because of the established functional importance of the amino-terminal amino acid sequences of Saccharomyces cerevisiae UBC9 and because of suggestions that the region of Arg8 to Phe24 might be involved in interactions with specific cellular targets (Yasugi and Howley, 1996). As a negative control, 35S-labeled proteins were incubated with GST protein alone bound to glutathione-Sepharose beads. As seen in Fig. 3A, 35S-labeled UBC9 bound specifically to GST-hBH, GST-DUBC9, and GST-hBH14–357. 35S-labeled DUBC9 also interacted with GST-hBH and GST-DUBC9 immobilized on glutathione-Sepharose beads (Fig. 3B, lanes 3 and 4), suggesting that the first 24 amino acids of hUBC9 were not essential for hBH interactions. Mutation of the active cysteine of UBC9 to tryptophan did not affect the coprecipitation of UBC9 with GST-hBH (Fig. 3C, lane 3).

**BH and UBC9 localization primarily in the cytoplasm and on the outer side of the nuclear membrane.** To investigate the possibility that hBH and UBC9 have similar locations, we analyzed transiently transfected CHO cells...
using indirect immunofluorescence. As shown in Fig. 4A, hBH showed a reticular pattern of staining, which surrounded the nucleus, extended through the cytoplasm, and appeared to be concentrated on the outside of (or to include) the nuclear membrane. No plasma membrane staining was observed, although there appeared to be faint staining of the nucleoplasm, as judged by the exclusion of the nuclei. The localization profile of hBH indicated that the enzyme is cytoplasmic and might be associated with the membranes of the endoplasmic reticulum and Golgi. A similar intracellular distribution pattern was shown in the cells overexpressing UBC9, although the staining was more diffuse and less intense in the nucleus. The same pattern of subcellular localization (especially the association with the nuclear envelope) was recently observed for endogenous UBC9, but with more protein residing in the nucleus, compared with the cytoplasm (Lee et al., 1998). We observed no specific staining when control nontransfected cells were treated using the same protocol (data not shown).

**hBH/hUBC9 interaction in vivo.** To confirm that interaction of hBH with hUBC9 occurred in vivo, we transiently transfected HEK293 cells with the pcDNA3.1 (+)Neo vector coding for hBH tagged with T7 epitope, with the pcDNA3.1 (+)His vector coding for hUBC9 tagged with AntiXpress epitope and six histidine residues, or with both. After 48 hr, the transfected cells were lysed and the supernatant was subjected to affinity purification on Ni-NTA-agarose. Bound material was eluted from Ni-NTA-agarose beads and examined by Western blotting using anti-T7 and AntiXpress antibodies. Nonpurified cell lysates from cells expressing hBH or UBC9 were loaded onto the gels as controls. Fig. 5 illustrates the affinity copurification results with HEK293 cell lysates from two independent transfections. As visible in the Western blots, epitope-tagged hBH migrated as a 70-kDa band (Fig. 5A, right) and epITOpe-tagged UBC9 migrated as a 50-kDa band (Fig. 5A, left). After Ni-NTA-agarose purification and Western immunoblotting of mock-transfected cell extracts, we found no immunoreactive material with anti-T7 or AntiXpress antibodies (Fig. 5B, lane 1). When expressed alone, hBH lacking the histidine tag failed to bind to Ni-NTA-agarose (Fig. 5B, lane 4). hBH was affinity purified on Ni-NTA-agarose when it was coexpressed with histidine-tagged hUBC9 (Fig. 5B, lane 4). These results demonstrated that hBH and UBC9 coprecipitate when they are coexpressed in mammalian cells.

**Evidence that overexpression of hBH does not change the SUMO-1 conjugation of cellular proteins.** UBC9 was recently shown to act as an E2-conjugating enzyme for the ubiquitin-like molecule SUMO-1. SUMO-1 was reported to modify RanGAP1, which is a small GAP; an acute promyelocytic leukemia-associated protein; and an unidentified set of nuclear proteins (Kamitani et al., 1997). SUMO-1-conjugated RanGAP1 appears in SDS-PAGE as an obvious band of 90-kDa (Mahajan et al., 1997). To determine whether hBH was involved in SUMO-1 conjugation of RanGAP1 or other cellular proteins, we fractionated lysates of wild-type CHO cells and CHO cells overexpressing hBH into cytosolic (S-100), membrane (P-100), and nuclear fractions and immunoblotted the fractions with an anti-SUMO-1 antibody. As shown in Fig. 6, high-molecular weight SUMO-1-conjugated proteins were observed in nuclear fractions of CHO cells and CHO cells overexpressing hBH into cytosolic (S-100), membrane (P-100), and nuclear fractions and immunoblotted the fractions with an anti-SUMO-1 antibody. As shown in Fig. 6, high-molecular weight SUMO-1-conjugated proteins were observed in nuclear fractions of CHO cells and CHO cells overexpressing hBH into cytosolic (S-100), membrane (P-100), and nuclear fractions and immunoblotted the fractions with an anti-SUMO-1 antibody. As shown in Fig. 6, high-molecular weight SUMO-1-conjugated proteins were observed in nuclear fractions of CHO cells and CHO cells overexpressing hBH into cytosolic (S-100), membrane (P-100), and nuclear fractions and immunoblotted the fractions with an anti-SUMO-1 antibody. As shown in Fig. 6, high-molecular weight SUMO-1-conjugated proteins were observed in nuclear fractions of CHO cells and CHO cells overexpressing hBH into cytosolic (S-100), membrane (P-100), and nuclear fractions and immunoblotted the fractions with an anti-SUMO-1 antibody.
we saw a similar band when lysates from mouse cells lacking the gene for BH were probed (data not shown). In general, there were no marked or reproducible differences in SUMO-1 conjugation of the observable cellular protein band between wild-type CHO cells and CHO cells overexpressing hBH, although we could not exclude the possibility that some high-molecular weight nuclear proteins were differentially affected.

**BH assays.** One of the possible consequences of a hBH/UBC9 interaction could be alteration of hBH function. Hydrolysis of the anticancer drug bleomycin is a unique property of BH that is not shared with any other known enzyme. Therefore, we examined the ability of hBH to degrade bleomycin when the two proteins were coexpressed. Cell lysates prepared from HEK293 and CHO cells transiently transfected with hBH, UBC9, or both were used to evaluate the hydrolysis of bleomycin. Lysates from cells transfected with UBC9 alone showed low levels of bleomycin A2 hydrolysis (<10%) associated with endogenous BH (Fig. 7). The bleomycin-hydrolyzing activity of cell lysates prepared from cells overexpressing hBH was increased 3- and 5-fold for HEK293 and CHO cells, respectively. Coexpression of hBH and UBC9 resulted in essentially no increase in bleomycin degradation. Similarly, we found that coexpression of UBC9Trp93 did not alter the BH activity measured in lysates. Therefore, we concluded that the bleomycin-hydrolyzing activity of hBH was not markedly changed as a result of coexpression with UBC9.

![Figure 4](image1) Figure 4. Indirect immunofluorescent localization of T7-hBH and Myc-hUBC9 expressed in CHO cells. CHO cells were transfected with pcDNA3.1hBHzeo (A) or pcDNA3.1UBC9MycHis (B) vectors and then processed for indirect immunofluorescence as described in Materials and Methods. A, T7-tagged hBH was visualized with anti-T7 primary and fluorescein isothiocyanate-conjugated secondary antibodies. B, Myc-tagged hUBC9 was immunostained with anti-Myc primary and Cy3-conjugated secondary antibodies. Slides were analyzed using Molecular Dynamics confocal laser-scanning microscopy.

![Figure 5](image2) Figure 5. hBH/UBC9 interaction in vivo. A, Western blots of total cell lysates from HER293 cells expressing UBC9 and probed with AntiXpress antibody (left) or expressing hBH and probed with anti-T7 antibody (right). Arrows, specific bands corresponding to the expected molecular masses. B, Western blots of Ni-NTA-agarose-bound and eluted material from cell lysates from cells expressing hBH, UBC9, or both. Cells were transfected with pcDNA3.1 vector (mock transfection) (lane 1), pcDNA3.1HisUBC9 and pcDNA3.1hBHzeo (lane 2), pcDNA3.1UBC9 (lane 3), or pcDNA3.1hBHzeo (lane 4). Immunoblotting was performed with anti-T7 antibody (upper) or AntiXpress antibody (lower).
Discussion

BH is an unusual multifunctional cysteine proteinase. BH is expressed in most tissues and has been well preserved during evolution, indicating an important but still poorly defined cellular role. In our attempts to understand the normal function of hBH, we searched for protein partners using a yeast two-hybrid system and identified the human homologue of UBC9. Although this approach has recently revealed partnerships between UBC9 and several biologically important proteins, including a tumor suppressor gene product (Wang et al., 1996), a transmembrane signaling protein (Wright et al., 1996), and a member of the nuclear transport machinery (Saitoh et al., 1997), hBH is the first proteinase partner. The interaction between hBH and UBC9 can be robustly reproduced both in vitro and in vivo.

What might be the consequences of an interaction between hBH and UBC9? An extensive series of experiments in this laboratory did not confirm the possibility of post-translational modification of hBH resulting from covalent binding of ubiquitin or SUMO-1, and the estimated half-life of hBH (>6 hr) does not support the idea of its intracellular fast proteasomal degradation (data not shown). Inhibition of other cysteine proteinases by endogenous proteins such as cystatins, which may be intra- or extracellular (those called stefins), and circulating kininogens is well known (Chapman et al., 1997). Therefore, the most obvious explanation could be that UBC9 physically binds to hBH and changes its enzymatic activity. The interaction between hBH and UBC9, however, did not require the last 100 amino acids, including the BHYD and two of the three catalytic amino acids. We previously demonstrated the requirement for the carboxyl terminus and the BHYD for the aminopeptidase and bleomycin-hydrolyzing activities (Koldamova et al., 1998). Most importantly, BH activity was not markedly decreased when UBC9 and hBH were coexpressed in mammalian cells. Therefore, physical inhibition of hBH activity seems extremely unlikely.

UBC9 is an essential gene in S. cerevisiae. Conditional ubc9 mutants are arrested in the cell cycle at G2/M and are impaired in proteolysis of B-type cyclins (Seufert et al., 1995), but a critical role for UBC9 as the conjugating enzyme involved in the ubiquitination of cyclin B has not been established. In contrast, there are accumulating biochemical data showing that UBC9 may act as an E2-conjugating enzyme for another ubiquitin-like molecule, SUMO-1. SUMO-1 modifies RanGAP1, a small GAP for Ran (required for nuclear transport), and this conjugation targets cytosolic RanGAP1 to RanBP2/Nup358, a component of the nuclear pore complex (Mahajan et al., 1997). Saitoh et al. (1997) found that the Xenopus laevis homologue of UBC9 forms a complex with both RanGAP1 and the binary complex of RanBP2 and the SUMO-1 conjugate of RanGAP1. UBC9 also forms a thioester with the SUMO-1 homologue Smt3p, but not with ubiquitin (Johnson and Blobel, 1997; Johnson et al., 1997). Therefore, UBC9 is the most probable candidate for transferring SUMO-1 and Smt3p to a substrate. There is no evidence that SUMO-1 conjugation targets any of these proteins for destruction; rather, SUMO-1-modified RanGAP1 seems to be more stable (Matunis et al., 1996; Mahajan et al., 1997). Therefore, it seems unlikely that UBC9 mediates the ubiquitin conjugation and proteasomal degradation of hBH. We have been unable to demonstrate altered SUMO-1 addition in the presence of hBH expression.

Another possible role for BH is to act as an adapter protein,

**Fig. 6.** Evidence that overexpression of hBH does not markedly change the SUMO-1 conjugation of cellular proteins. Western blot analysis was performed with subcellular fractions of wild-type CHO and CHO cells overexpressing hBH. The nuclear fraction (Nuc), cytosolic (S-100) fraction, and membrane (P-100) fraction were prepared as described in Materials and Methods and were analyzed by Western blotting using an anti-SUMO-1 antibody. Arrow, RanGAP1 modified with SUMO-1 (p90). Molecular mass standards (left) are expressed in kilodaltons.

**Fig. 7.** BH assays of lysates from HEK 293 and CHO cells transfected with UBC9, hBH, or both. BH activity was determined in lysates prepared from CHO (A) or HEK293 (B) cells expressing UBC9 (1), hBH (2), or both (3). The assays were performed as described in Material and Methods. Bleomycin-hydrolyzing activity is presented as a percentage of the total amount of bleomycin A2 (A2) undergoing degradation.
mediating interactions between UBC9 and other proteins. yBH has been co-localized and co-purified with Gce1p, a cAMP-binding ectoprotein that is associated with the plasma membrane by a glycosyl-phosphatidylinositol anchor (Magdolen et al., 1993; Niemer et al., 1997). Kambouris et al. (1992) isolated BLH1/yBH as an amphotropic protein occurring both in the cytoplasm and bound to the plasma membrane. The regulatory activity of yBH seems to be independent of both the protease and DNA-binding activities and could reflect interactions with other protein partners (Zheng et al., 1997). The ability of hBH to interact with UBC9 may provide an explanation for the preservation of BH through evolution and its ubiquitous expression in mammalian cell types.

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References


