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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-napthylamide-hydrolyzing activity. Magdolen *et al.* (1993) copurified yBH with Gce1p, 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 active sites in 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

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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_2, deamidobleomycin A_2; 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; Ni-NTA, Ni^{2+} 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, cyh^{r}2, LYS2::GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*). Y190 cells co-transformed with pVA3 and pTD served as positive controls. We confirmed the expression of the hBH fusion protein by Western immunoblotting with anti-GAL4 binding domain monoclonal antibodies (Clontech). We determined β-galactosidase activity after transferring the yeast colonies to Whatman filters, lysing the submerged filters in liquid nitrogen, and thawing the filters at room temperature. In general, β-galactosidase activity was apparent within 1 hr, but the filters were allowed to incubate for 8 hr.

To generate some of the recombinant expression vectors, we used a previously described hBH cDNA (Koldamova *et al.*, 1998), which contained T7 epitope sequences (Novagen, Madison, WI). For *in vitro* transcription and translation, we used pCite4 vectors (Novagen) for optimal expression of cloned inserts. We applied the prokaryotic expression vector pTrcHisC (Invitrogen, Carlsbad, CA) to express fusion proteins with six histidine residues for further purification using Ni-NTA-agarose (Qiagen, Santa Clarita, CA). The HeLa library true-positive clone pGADgh113 1, containing full-length cDNA for the human homologue of UBC9, was used as a template for the restriction enzyme digestions and polymerase chain reaction amplifications. pcDNA3.1His and pcDNA3.1(+)Zeo mammalian expression vectors (Invitrogen) were used to subclone hBH, hUBC9, or corresponding mutants and truncated forms. The following recombinant vectors were generated as previously described (Koldamova *et al.*, 1998): pAS2–1hBH, pAS2–1hBH$_{14–289}$, pAS2–1hBH$_{1–175}$, pAS2–1hBH$_{1–357}$, and pAS2–1hBH$_{194–357}$ for yeast two-hybrid screening and analyses; pGEX4ThBH, pGEX4TΔBH, and pGEX4ThBH$_{14–357}$ for *in vitro* binding assays.

The entire cDNA for UBC9, including 76 nucleotides (in-frame) preceding the first ATG codon and a 3′-untranslated region consisting of 541 nucleotides and ending after a putative polyadenylation signal, was excised from pGADgh113 1 using *Bam*HI and *Xho*I restriction enzymes and was subcloned into pCite4, thus generating the pCite4UBC9+ vector for *in vitro* transcription/translation and binding experiments. An amino-terminally truncated form of UBC9 without the first 24 amino acids (pCite4ΔUBC9) was generated by polymerase chain reaction amplification using the following forward and reverse primers (the restriction enzyme recognition sites within the primers are underlined): 5′-CATGTGAATTCGTGGCTGTC-3′ (forward) and 5′-AAGGAAGATCTGCTTAGGAGGACG-3′ (reverse). 5′-CGGAATTCATGTCGGGGATCGCT-3′ (forward) and 5′-AAGGAAGATCTGCTTAGGAGGACG-3′ (reverse) were the primers used to generate the pCite4UBC9 recombinant vector without additional 5′ and 3′ sequences present in the original library cDNA. The *in vitro* UBC9 products were synthesized according to the manufacturer's directions for the TnT rabbit reticulocyte lysate system (Promega).

We used the following nucleotide sequence as a forward primer to generate full-length hBH for subcloning into pcDNA3.1Zeo: 5′-CCGAAGCTTGACCATGGCCAGTATGAC-3′. This primer contained a *Hin*dIII recognition site (underlined) as a 5′ extension and a functional ATG translation start within a Kozak consensus sequence (double-underlined) (Kozak, 1987). The functional ATG codon was designed to be the translation start of the T7 epitope (Novagen), consisting of 11 amino acids and fused to the amino terminus of hBH. 5′-GCGCGGATCCAGTATCACTCACTCAGCCAA-3′ was the corresponding reverse primer. hUBC9 was subcloned into the pcDNA3.1His mammalian expression vector (Invitrogen) by transferring the entire library cDNA from pGADgh113 1 as a *Bam*HI-*Xho*I fragment, thus generating pcDNA3.1UBCHis. For subcloning into the pcDNA3.1MycHis vector (Invitrogen), we used the following primers: 5′-GCGAATTCAACATGGCGGGGAT-3′ (forward) and 5′-GGCAAGCTTCGTATAGAGGGCGCAAACT-3′ (reverse). The primers made possible amplification of a full-length hUBC9 cDNA with its own ATG start codon, within a Kozak consensus sequence, and a mutation from adenine to cytosine within the stop codon, generating tyrosine as amino acid 159. This ensured in-frame fusion of the hUBC9 carboxyl terminus to the MycHis epitope sequence and generation of the pcDNA3.1UBC9MycHis vector. We used the same forward and reverse primers to amplify and subsequently clone hUBC9$_{Cys93→Trp93}$ (see below) into pcDNA3.1MycHis using the mutated pCite4UBC9 as a template, thus generating pcDNA3.1MycHisUBC9T$_{93}$.

Site-directed mutagenesis. Oligonucleotide-directed, site-specific mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene, San Diego, CA). Oligonucleotides complementary to both strands of hUBC9 were synthesized to change the active Cys93 to Trp93, as follows: 5′-GGAACAGTGTGGCTGAGCATCTTAG-3′ (forward) and 5′-CTAAGATGCTCAGCCACACTGTTCC-3′ (reverse). The reaction mixtures (50-μl final volume) consisted of 10 ng of double-stranded DNA vector (either pCite4UBC9+ or pCite4UBC9), 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 *Dpn*I restriction enzyme, to digest the parental, nonmutated, supercoiled, double-stranded DNA. We used 2 μl of *Dpn*I –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 (hBH$_{14–455}$), or hBH$_{14–357}$ were expressed in *Escherichia coli* DH5α and affinity-purified on glutathione-Sepharose (Pharmacia) as described previously (Koldamova *et al.*, 1998). Briefly, ^{35}S-labeled UBC9, ΔUBC9, or UBC9Trp93 (3 μl) was incubated at 4° for 1 hr with the GST-fusion constructs of hBH bound to glutathione-Sepharose beads (25 μl), in 50 mM NaCl with 1 mg/ml bovine serum albumin. 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 on SDS-polyacrylamide 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. 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% CO_2. 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

(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-cm^2 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 × 10^6 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 aprotinin). Cells were sonicated, and lysates were cleared of nuclei and debris by centrifugation at 14,000 × *g* at 4° for 10 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°. The bound proteins were washed five times and eluted with SDS sample buffer (0.25 M 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 horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (diluted 1/3,000). Signals were detected using a chemiluminescence detection assay (NEN, Boston, MA) and a <1-min exposure to X-ray film.

Subcellular fractionation. Subcellular fractionation of CHO cells and CHO cells transfected with pcDNA3.1hBHZeo was performed as described previously (Kamitani *et al.*, 1997). Briefly, to prepare S-100 and P-100 fractions, 3 × 10^7 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 $MgCl_2$, 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 × 10^7 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 pcDNA3.1hBHZeo) 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 A_2 from its inactive metabolite dA_2 (Sebti *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 μM bleomycin A_2 (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 $CuSO_4$, the mixture was centrifuged, and the resulting supernatant fractions were injected onto a C_8 reverse-phase high performance liquid chromatography column (3.9 mm × 150 mm, 5-μm particle size; Waters Chromatography, Milford, MA). Bleomycin A_2 and dA_2 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 A_2 converted into dA_2 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 10^6 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 (ΔBH), 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 $hBH_{1–357}$, 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).

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AGCGCCGCCGCCCCGCTCGGTCCTCCACCTGTCCGCTACGCTCGCCGGGGCTGCGGCCGCCCGAGGGACTTTGAAC
ATG TCG GGG ATC GCC CTC AGC AGA CTC GCC CAG GAG AGG AAA GCA  45
 M   S   G   I   A   L   S   R   L   A   Q   E   R   K   A 15

TGG AGG AAA GAC CAC CCA TTT GGT TTC GTG GCT GTC CCA ACA AAA  90
 W   R   K   D   H   P   F   G   F   V   A   V   P   T   K 30

AAT CCC GAT GGC ACG ATG AAC CTC ATG AAC TGG GAG TGC GCC ATT  135
 N   P   D   G   T   M   N   L   M   N   W   E   C   A   I 45

CCA GGA AAG AAA GGG ACT CCG TGG GAA GGA GGC TTG TTT AAA CTA  180
 P   G   K   K   G   T   P   W   E   G   G   L   F   K   L 60

CGG ATG CTT TTC AAA GAT GAT TAT CCA TCT TCG CCA CCA AAA TGT  225
 R   M   L   F   K   D   D   Y   P   S   S   P   P   K   C 75

AAA TTC GAA CCA CCA TTA TTT CAC CCG AAT GTG TAC CCT TCG GGG  270
 K   F   E   P   P   L   F   H   P   N   V   Y   P   S   G 90

ACA GTG TGC CTG TCC ATC TTA GAG GAG GAC AAG GAC TGG AGG CCA  315
 T   V   C   L   S   I   L   E   E   D   K   D   W   R   P 105

GCC ATC ACA ATC AAA CAG ATC CTA TTA GGA ATA CAG GAA CTT CTA  360
 A   I   T   I   K   Q   I   L   L   G   I   Q   E   L   L 120

AAT GAA CCA AAT ATC CAA GAC CCA GCT CAA GCA GAG GCC TAC ACG  405
 N   E   P   N   I   Q   D   P   A   Q   A   E   A   Y   T 135

ATT TAC TGC CAA AAC AGA GTG GAG TAC GAG AAA AGG GTC CGA GCA  450
 I   Y   C   Q   N   R   V   E   Y   E   K   R   V   R   A 150

CAA GCC AAG AAG TTT GCG CCC TCA TAA GCAGCGACTTGTGGCATCGTCAGA 477
 Q   A   K   K   F   A   P   S   -                          158
GGAAGGATGGTTTGGCAAGAATTGTTTACAACATTTTTGCAAATCTAAAGTTGACTCCATACAATGACTANTCACC
TGGGGGGGTTGGGCGGGCGCCATCTTCCATTGCCGCCGCGGGTGTGCGGTCTCGATTCGCTGAATTGCCCGTTTCC
ATACAGGGTCTCTTCCTTCGGTCTTTTGTATTTTTGATTGTTATGTAAAACTCGCTTTTATTTTAATATTGATGTC
AGTATTTCAACTGCTGTAAAATTATAAACTTTTATACTTGGGTAAGTCCCCCAGGGGCGANTTCCTCGCTCTGGGA
TGCANGCATGCTTCTCACCGTGCANAGCTGCACTTGGCCTCAGCTGGCTGTATGGAAATGCACCCTCCCTCCTGCC
GCTCCTCTCTCTAGAACCTTCTAAAACCTGGGCTGTGCTGCTTTTGAACCTCAGACCCCAGGGCAGCATCTCGGTTCT
GCGCCACTTCCTTTGTGTTTATATGGCNTTTTGTCTGTGTTGCTGTTTAAAATAAATAAACTGTTTATATAAAAAA
AAAAAAAAAAAAAAACTCGAACNCCACCACCAACCACCACTGAAAT
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Fig. 1. Human homologue of yeast UBC9. The nucleotide and deduced amino acid sequences of hUBC9 (including 5′- and 3′-untranslated regions), as found in the pGADgh113 1 HeLa library positive clone, are shown. The ATG start codon and the TAA stop codon (positions +1 and +475, respectively) are *double-underlined*. A presumed polyadenylation signal is *italic, bold, and underlined*.

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 ^{35}S-labeled proteins corresponding to the full-length coding sequence of UBC9, an amino-terminal deletion mutant without the first 24 amino acids (ΔUBC9), or full-length UBC9 in which the active residue Cys93 had been mutated to tryptophan (UBC9Trp93). We generated ΔUBC9 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, ^{35}S-labeled proteins were incubated with GST protein alone bound to glutathione-Sepharose beads. As seen in Fig. 3A, ^{35}S-labeled UBC9 bound specifically to GST-hBH, GST-ΔBH, and GST-hBH$_{14–357}$. ^{35}S-labeled ΔUBC9 also interacted with GST-hBH and GST-ΔBH 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

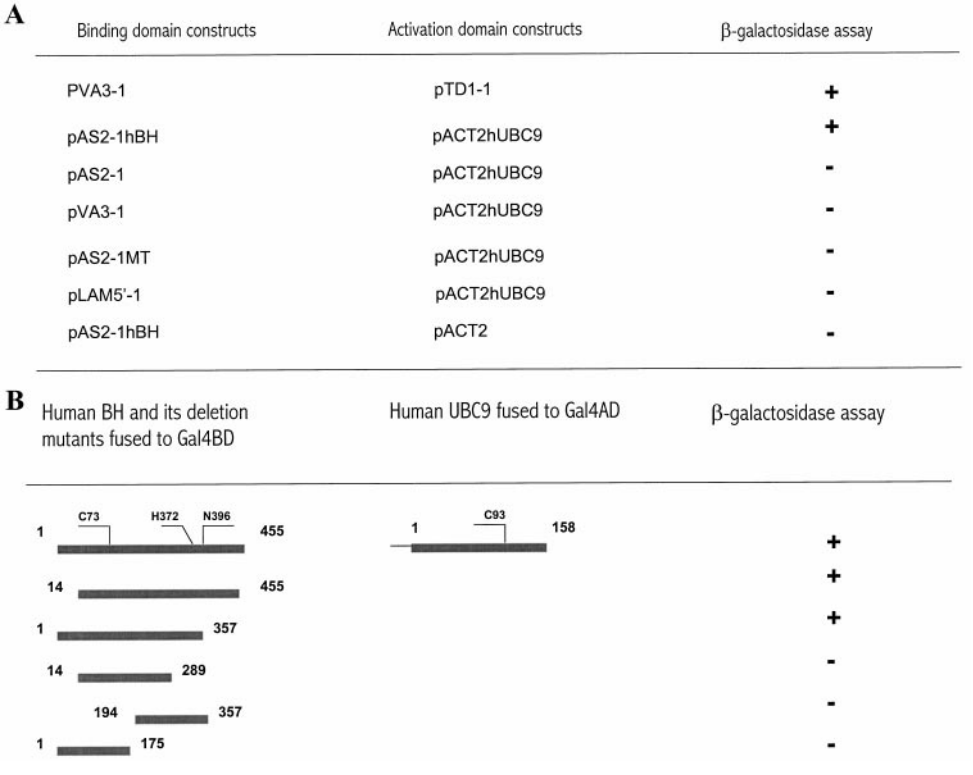

Fig. 2. hBH/UBC9 interaction in a two-hybrid assay. A, To test the specificity of the hBH/UBC9 interaction in the yeast two-hybrid assay, UBC9 was coexpressed with different unrelated proteins, namely murine p53 (pVA3–1), metallothionein (pAS2–1MT), lamin (pLAM5–1), and the binding domain of GAL4 (pAS2–1). The interaction of p53 and the simian virus 40 large T antigen (pTD1–1) was used as a positive control. β-Galactosidase activity was monitored by using the qualitative filter assay, as described in Materials and Methods (+, development of blue color; −, no color development). B, Yeast strain Y190 cells were transformed with different combinations of hBH deletion mutants fused to the GAL4 binding domain (*Gal4BD*) in pAS2–1 and hUBC9 fused to the GAL4 activating domain (*Gal4AD*) in pACT2 plasmids.

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 outer side 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 nucleoli. 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).

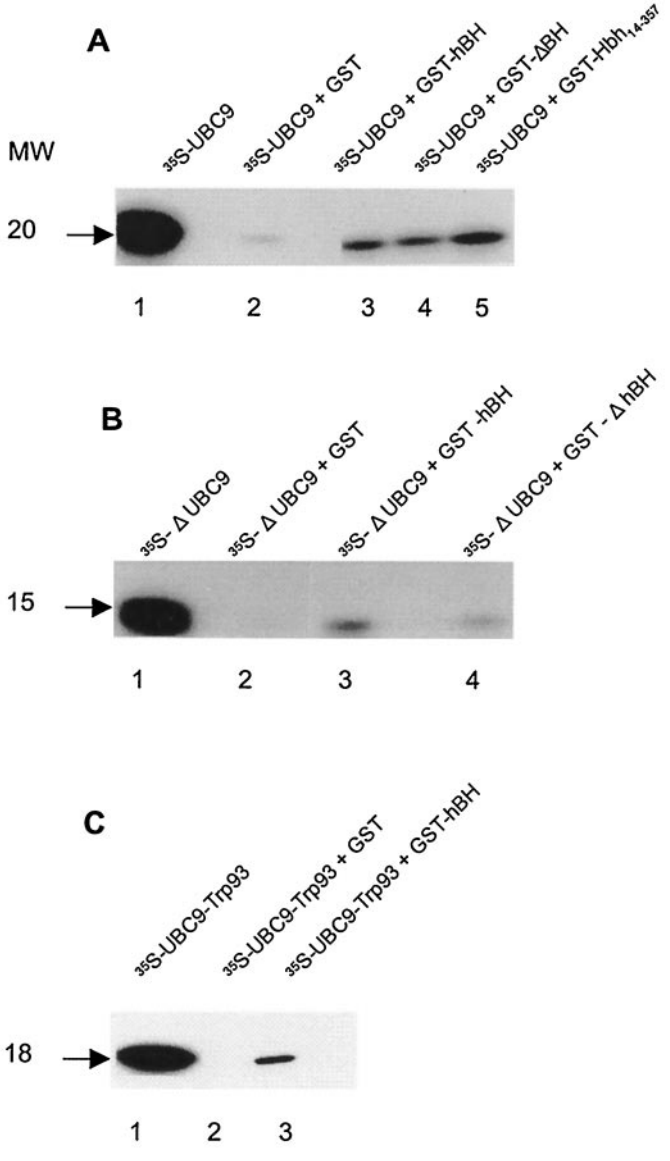

Fig. 3. GST-hBH binding *in vitro* with ^{35}S-labeled UBC9, ^{35}S-labeled ΔUBC9, or ^{35}S-labeled UBC9Trp93. A, ^{35}S-labeled full-length UBC9 (*lane 1*) was precipitated with GST-tagged hBH (*lane 3*), ΔBH (hBH_{14-455}) (*lane 4*), or hBH_{14-357} (*lane 5*) but not with GST alone (*lane 2*). B, ^{35}S-labeled ΔUBC9 (*lane 1*) was precipitated with GST-tagged hBH (*lane 3*) and ΔBH (*lane 4*) but not with GST (*lane 2*). C, ^{35}S-labeled UBC9Trp93 (*lane 1*) was precipitated with GST-tagged hBH (*lane 3*) but not with GST alone (*lane 2*). Precipitated proteins were separated on 12% SDS-polyacrylamide gels, which were dried and exposed to X-ray film.

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(+)Zeo vector coding for hBH tagged with T7 epitope, with the pcDNA3.1(+)His vector coding for hUBC9 tagged with Anti-Xpress 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 ~50-kDa band (Fig. 5A, *right*) and epitope-tagged UBC9 migrated as a ~30-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 only 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 (Fig. 6, *lanes 1* and *4*); similar results were reported by other investigators (Lee *et al.*, 1998). Membrane (P-100) fractions of CHO cells and CHO cells overexpressing hBH (Fig. 6, *lanes 3* and *6*) contained a predominant 90-kDa band, corresponding to RanGAP1 modified by SUMO-1, as reported by others (Lee *et al.*, 1998). A less intense, 90-kDa band was visible in cytosolic (S-100) fractions of both CHO cells and CHO cells overexpressing hBH (Fig. 6, *lanes 2* and *5*). A second, more intense, band at 70 kDa was also seen in the cytosolic fractions. Although in some studies the P-100 fractions of CHO cells overexpressing hBH contained slightly more of the 70-kDa protein than did wild-type cells, this was not a reproducible finding. Based on studies with antibodies against BH, we concluded that the 70-kDa band was not BH modified with SUMO-1. Moreover,

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.

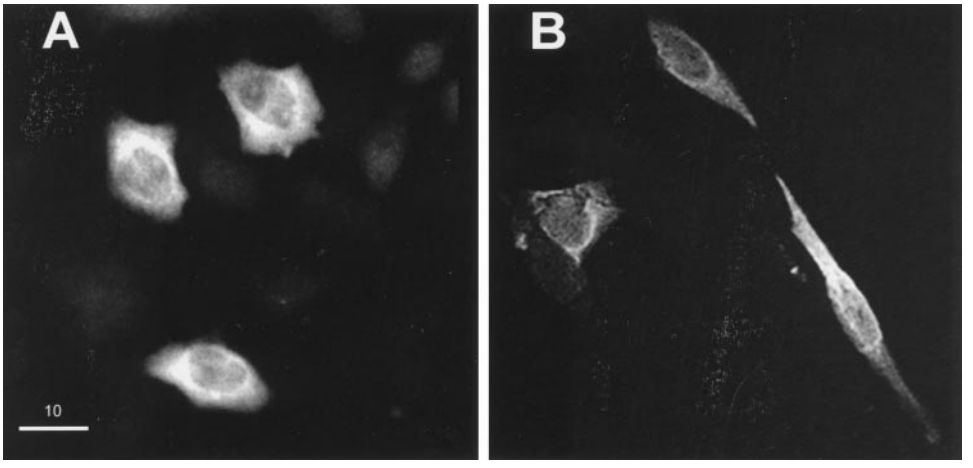

Fig. 4. Indirect immunofluorescent localization of T7-hBH and Myc-hUBC9 expressed in CHO cells. CHO cells were transfected with pcDNA3.1hBHZeo (A) or pcDNA3.1UBC9MycHis (B) vectors and then processed for indirect immunofluorescence as described in Materials and Methods. A, T7-tagged hBH was visualized with anti-T7 primary and fluorescein isothiocyanate-conjugated secondary antibodies. B, Myc-tagged hUBC9 was immunostained with anti-Myc primary and Cy-3-conjugated secondary antibodies. Slides were analyzed using Molecular Dynamics confocal laser-scanning microscopy.

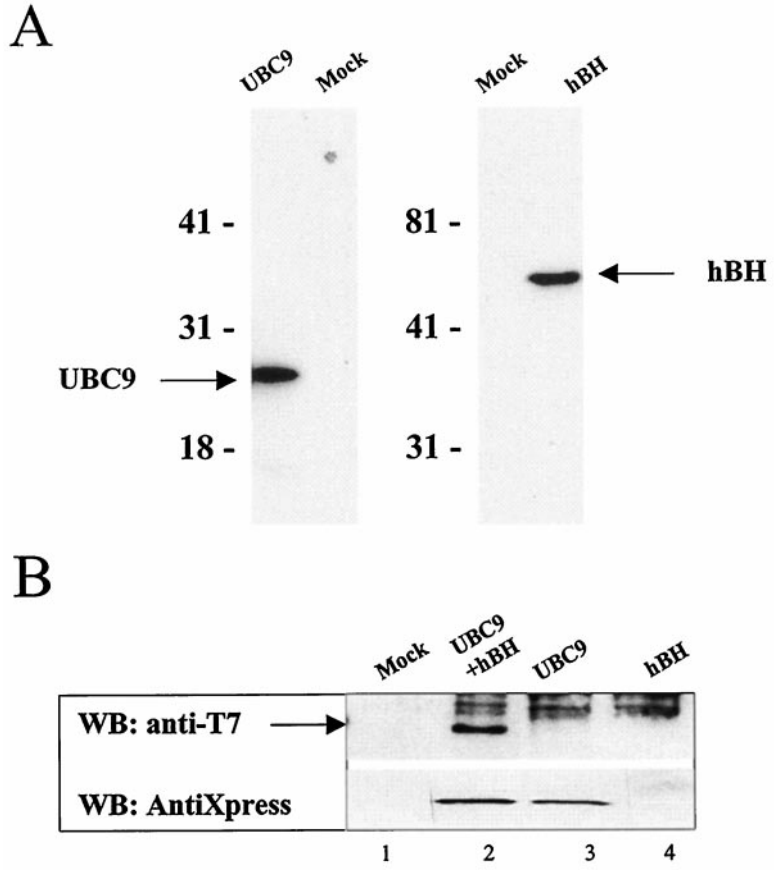

Fig. 5. hBH/UBC9 interaction *in vivo*. A, Western blots of total cell lysates from HEK293 cells expressing UBC9 and probed with AntiXpress antibody (*left*) or expressing hBH and probed with anti-T7 antibody (*right*). *Arrows*, specific bands corresponding to the expected molecular masses. B, Western 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 proteases 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.

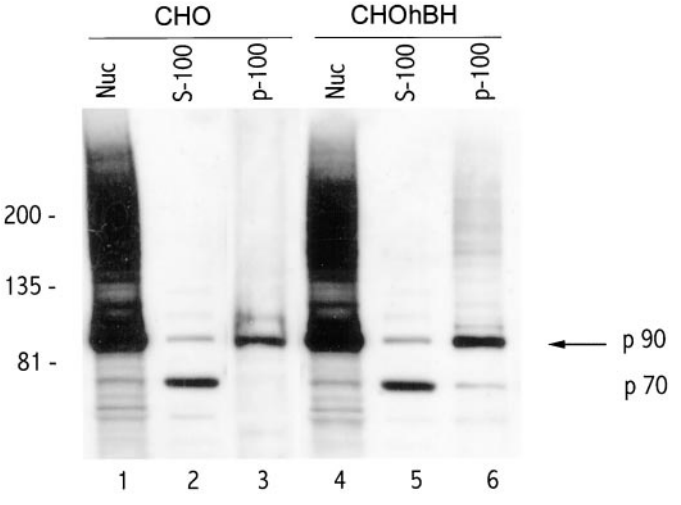

Fig. 6. Evidence that overexpression of hBH does not markedly change the SUMO-1 conjugation of cellular proteins. Western blot analysis was performed with subcellular fractions of wild-type CHO and CHO cells overexpressing hBH. The nuclear fraction (*Nuc*), cytosolic (S-100) fraction, and membrane (P-100) 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.

UBC9 is an essential gene in *S. cerevisiae*. Conditional *ubc9* mutants are arrested in the cell cycle at G_2/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,

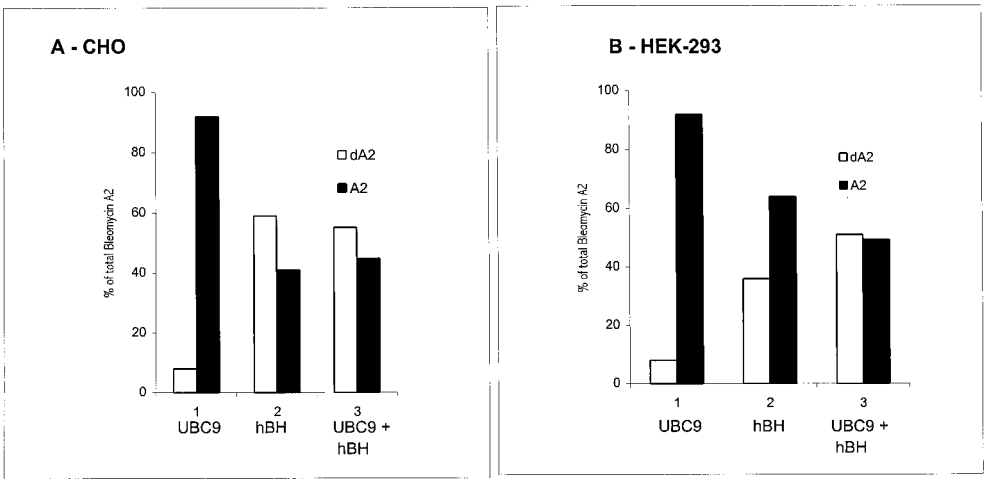

Fig. 7. BH assays of lysates from HEK 293 and CHO cells transfected with UBC9, hBH, or both. BH activity was determined in lysates prepared from CHO (A) or HEK293 (B) cells expressing UBC9 (*1*), hBH (*2*), or both (*3*). The assays were performed as described in Material and Methods. Bleomycin-hydrolyzing activity is presented as a percentage of the total amount of bleomycin A_2 (*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 throughout evolution and its ubiquitous expression in mammalian cell types.

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