Human Cystathionine β-Synthase Is a Target for Sumoylation

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ABSTRACT: Cystathionine β-synthase (CBS) catalyzes the first irreversible step in the transsulfuration pathway and commits the toxic metabolite, homocysteine, to the synthesis of cysteine. Mutations in CBS are the most common cause of severe hereditary hyperhomocysteinemia. The molecular basis of the organ-specific pathologies associated with CBS deficiency is unknown as is the significance of the reported interaction between CBS and Huntingtin protein. In this study, we have used the yeast two-hybrid approach to screen for proteins that interact with CBS and have identified several components of the sumoylation pathway including Ubc9, PIAS1, PIAS3, Pc2, and RanBPM. We demonstrate that CBS is modified by the small ubiquitin-like modifier-1 protein (SUMO-I) under both in vitro and in vivo conditions. Deletion analysis of CBS indicates that the C-terminal regulatory domain is required for interaction with proteins in the sumoylation machinery. Sumoylated CBS is present in the nucleus where it is associated with the nuclear scaffold. The discovery that CBS is a target of sumoylation adds another layer to the complex regulation of this enzyme and reveals a previously unknown residence for this protein, i.e., in the nucleus.

Cystathionine β-synthase (CBS) catalyzes the pyridoxal 5′-phosphate-dependent condensation of serine and homocysteine to form cystathionine, the first committed step in the disposal of homocysteine via the transsulfuration pathway (*1*, *2*). In mammals, CBS is a highly regulated enzyme, which contains a heme cofactor that functions as a redox sensor (*3*) and utilizes AdoMet as an allosteric activator (*4*). In addition, the C-terminal domain of CBS contains a tandem repeat of two "CBS domains" and exerts intrasteric regulation which is alleviated by truncation of this domain (*5*−*7*). Although the exact function of the "CBS domain" is not known, it is proposed to function as an energy-sensing module (*8*). Mutations in this domain are correlated with hereditary diseases (*9*−*11*).

Elevated levels of homocysteine are correlated with cardiovascular diseases, neural tube defects, and neurodegenerative diseases, viz., Parkinson's disease and Alzheimer's disease (*12*−*14*). It is not known if homocysteine is a cause or a consequence of these pathologies. CBS deficiency affects four major organ systems including the ocular, cardiovascular, skeletal, and central nervous systems. Mutations in CBS are the single most common cause of severe hereditary hyperhomocysteinemia, and over 100 mutations have been described at this locus (*15*). A subset of these mutations exhibits robust activity when expressed in recombinant form (*16*, *17*). The existence of these mutants raises the intriguing possibility that their critical defects may lie not in the ability to catalyze the reaction but, instead, in the ability to engage in essential intermolecular interactions. Interaction of CBS with the Huntingtin protein has been reported and was in fact identified in a yeast two-hybrid screen using Huntingtin as the bait (*18*). While the significance, if any, of this interaction to the function of either protein is unknown, it has been suggested that oxidation products of homocysteine may be involved in Huntington's disease-associated death of striatal neurons that is known to be partly mediated by excitotoxic insult (*19*).

Homozygous disruption of the CBS gene in mice results in severely elevated plasma homocysteine concentrations and greatly reduced viability (*20*). In an interesting recent study, expression of a mutant human CBS transgene was shown to rescue neonatal lethality but not hyperhomocysteinemia in mice (*21*). The human CBS transgene harbored a common pathogenic mutation, I278T, which led to the expected very low level of enzyme activity and was consistent with the presence of high homocysteine in these mice. On the basis of these observations, the authors suggested that CBS may have a function in addition to its enzymatic activity in homocysteine catabolism that is important particularly during development.

Protein modifications are both numerous in kind and in the responses they elicit in function, activity, or localization. They range from small molecule modifications, e.g., methylation and phosphorylation, to large covalent tags, e.g., ubiquitination and sumoylation. Sumoylation of the Huntingtin protein competes with ubiquitination and inhibits aggregation of a pathogenic fragment (*22*). SUMO is a small ubiquitin-like modifier protein of ∼11 kDa molecular mass that is structurally similar to ubiquitin. Sumoylation often occurs at lysine residues embedded in a consensus sequence, ΨKXE, in which Ψ is a hydrophobic amino acid and X is any amino acid. This modification can elicit pleiotropic effects that include a change in subcellular localization typically involving movement to the nucleus, protein partnering, and modulating the DNA-binding and/or transacti-

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vation activities of transcription factors (*23*).

In this study, we have used a yeast two-hybrid approach to screen for interacting proteins that could potentially illuminate more fully the function of CBS in vivo and perhaps explain the etiology of organ-specific deficits associated with pathogenic mutations in this gene. This work has led to the identification of a new modification of CBS, i.e., sumoylation, and revealed a previously unknown residence for this protein, i.e., in the nucleus.

MATERIALS AND METHODS

Materials. Trypsin was purchased from Gibco BRL and Protein A−Sepharose CL-4B and glutathione−Sepharose beads were purchased from Amersham Biosciences. Aos1/Uba2, Ubc9, and SUMO-1 were purchased from LAE Biotech International. All other chemicals were purchased from Sigma.

Construction of Plasmids. The yeast two-hybrid vector pGBDU-C2 was kindly provided by Dr. Elizabeth Craig, University of Wisconsin, Madison. pGBDU-C2/hCBS (containing full-length CBS) and pGBDU-C2/CBS-ΔN69 (lacking the N-terminal heme domain) were generated by cloning the respective gene fragments, generated by digestion of parent plasmids pGEX4-T1/hCBS (*7*) and pGEX4-T1/CBS-ΔN69 (*3*) with *Sal*I, and ligating to *Sal*I-digested pGBDU-C2. pGBDU-C2/CBS-ΔC143 was constructed by inserting the *Eco*RI fragment of pGEXCBSN, expressing the ΔC143 variant of CBS (*7*), into the *Eco*RI site of pGBDU-C2. pGBDU-C2/CBS-ΔN336, expressing the C-terminal 215 residues, was constructed in two stages. First, a 1012 bp fragment from the 5′ end of the CBS gene was excised by digesting the pGEX4-T1/CBS plasmid with *Sph*I and religating the linearized vector to give pGEX4-T1/CBS-ΔN336. The latter was then digested with *Sal*I, and the resulting fragment was cloned into the *Sal*I site of pGBDU-C2. To construct the pGBDU-C2/CBS-ΔN411 vector expressing the C-terminal 139 residues, a 420 bp fragment from the 3′ end of the CBS coding sequence was amplified by PCR using a sense primer, 5′-CCCTGGTGGTGGC**GCATGC**GTGTTCAGGAGC-3′ (containing a *Sph*I site shown in bold), and an antisense vector-specific primer, 5′-CAGTCACGATGCGGCCGCTCGA-3′. The PCR product was digested with *Sph*I and *Xho*I, and the gene-cleaned fragment was ligated to the fragment generated by digesting pGEX4-T1/CBS with *Sph*I and *Xho*I giving pGEX4-T1/CBS-ΔN411. pGEX4-T1/CBS-ΔN411 was digested with *Sal*I, and the gene-cleaned fragment was cloned into pGBDU-C2 linearized with the same enzyme. In the pGEX4-T1/CBS-ΔN411 construct, a new ATG start codon was introduced by the *Sph*I site and replaced the CTC codon encoding L412 in full-length CBS. The sequences of all constructs generated using a PCR step were verified by nucleotide sequence determination at the Genomics Core Facility at the University of Nebraska, Lincoln.

A human brain *Xho*I-oligo dT-primed cDNA library fused to the GAL4 activator domain of the prey plasmid pACT2 (carrying a *LEU2* marker gene) in *Escherichia coli* was purchased from Clontech. The library was amplified according to the manufacturer's protocol, and the plasmid library was isolated using Clontech's NukleoBond mega plasmid purification kit.

Yeast Strain. The reporter yeast strain PJ694A/α containing three reporter genes, *LEU2*, *HIS3*, and *lacZ*, under the control of separate promoters described elsewhere (*24*) was employed in the yeast two-hybrid screen. The genotype of the yeast was *MAT*a *trp1-901 leu2-3, 112 ura3-52 his3-200 gal4*Δ *gal180*Δ *LYS::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*.

Yeast Transformations and Two-Hybrid Screening. Yeast mating was used to introduce the bait and the prey plasmids into the host cell. Yeast cells, PJ694 (MATα) and PJ694 (MATA), were transformed with the bait plasmid and the pACT2/cDNA library, respectively, using the lithium acetate method (*25*). Transformed cells were selected on synthetic minimal agar media that lacked uracil (SD-ura) for the selection of the bait, pGBDU-C2/CBS, and leucine (SD-leu) for the selection of the library plasmid, pACT2/cDNA. Mating was performed on YAPD media for 7 h, and cells were transferred to SD-leu/-ura plates to select for double transformants. Approximately 1.9×10^6 colonies were screened for the expression of three reporter genes, ADE2, HIS3, and β-galactosidase. Plasmids were isolated from the colonies that tested positive for the three reporter genes and analyzed by DNA sequencing using the pACT2 vector-specific primer, 5′-CTATTCGATGATGAAGATACCCCACCAAACCC-3′.

Protein Expression and Purification. Wild-type and mutant (D444N or P78R/K102N) human CBS was expressed and purified as described previously (*26*). pGEX4-T1/mPIAS1 expressing the full-length protein inhibitor of activated STAT1 (PIAS1) protein, an E3 SUMO ligase, was kindly provided by Dr. Ke Shuai (UCLA). PIAS1 was expressed and purified from *E. coli* BL21 cells (Invitrogen) induced with 0.1 mM IPTG at 25 °C for 14 h. The protein was purified by affinity chromatography on GST−Sepharose beads. The fusion protein was subsequently cleaved by thrombin, and the GST was separated from PIAS1 by passage through a second GST column as described previously (*27*).

In Vitro Sumoylation. In vitro sumoylation of CBS was performed in a 50 μL reaction volume containing 63 nM Aos1/Uba2, 3 μM Ubc9, 4.5 μM SUMO-I, 5 μM PIAS1, 5 mM ATP, 5 mM $MgCl_2$, 50 mM Tris·HCl, pH 7.5, and 6.5 μM purified recombinant CBS (wild type or the pathogenic mutants, D444N or P78R/K102N). Rabbit reticulocyte lysate (RRL), 20 μL (Promega), was added where indicated. Reaction mixtures were incubated at 37 °C for 2 h. Sumoylated CBS was immunoprecipitated (see below) with anti-SUMO-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The immunoprecipitate was analyzed by SDS−PAGE and detected using anti-CBS antibody.

Cell Culture. HepG2 cells and the neuroblastoma cells, SH-SY5Y, were from American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C, under 5% CO_2.

Preparation of Cell Extracts and Western Blotting. Cells were trypsinized and harvested by centrifugation at 1500*g*, washed twice with PBS, and lysed in buffer P containing 100 mM phosphate, pH 7.4, 150 mM $NaCl_2$, 0.1% Triton X-100, 0.1 mM PMSF, 1 μg/mL pepstatin A, and 1 μg/mL leupeptin. Where indicated, SUMO isopeptidase inhibitors, iodoacetic acid (IAA) and *N*-ethylmaleimide (NEM), were added to the lysis buffer at 250 and 800 μM final concentra-

tion, respectively. Cells were lysed for 30 min on ice with three freeze−thaw cycles. The sample was cleared by centrifugation at 14000*g* for 10 min at 4 °C. For brain tissue extract, mouse or pig brains, powdered in liquid nitrogen, were suspended in buffer P and lysed as described above. Alternatively, the brain powder was directly suspended in SDS loading buffer at 85 °C and boiled immediately.

Rabbit polyclonal anti-SUMO-I antibody (Santa Cruz Biotechnology) and chicken polyclonal anti-CBS antibody (*28*) were used for Western blot analysis. Detection of horseradish peroxidase-conjugated secondary antibodies was performed using the chemiluminescent peroxidase substrate (Sigma) according to the manufacturer's protocol.

Immunoprecipitation. Anti-SUMO-1 or anti-CBS antibody was added to cell extracts and incubated on a rotary shaker at 4 °C for 3 h. Protein A−Sepharose beads were added, and incubation was continued for 3 h. Following centrifugation at 5000*g* for 1 min, the resulting Sepharose pellets were washed five times with buffer P. The Sepharose pellet from the last wash was denatured by boiling in SDS sample buffer to solubilize the proteins.

Nuclear Isolation. One gram of liver or brain tissue powdered in liquid nitrogen was dissolved in 10 mL fo PBS containing 0.1% Triton X-100 and 2 mM DTT and homogenized on ice for 30 s using a hand-held homogenizer (Omni International, Marietta, GA) set at medium speed. Lysis was continued by incubating on ice and checked by using the trypan blue dye on an aliquot of the sample. Nuclei were pelleted by centrifugation at 1000*g* for 10 min at 4 °C. The nuclear pellet was dissolved in 10 mL of lysis buffer, and the nuclei were further purified from the membranous contaminants by passing through a 1.8 M sucrose cushion solution (SCS) using a Nuclei PURE Prep nuclei isolation kit (Sigma-NUC-2001). Briefly, the suspension of nuclei was mixed with 18 mL of 1.8 M SCS and layered on 10 mL of 1.8 M SCS in ultracentrifuge tubes (1 × 3.5 in, Beckman) and centrifuged at 30000*g* for 45 min at 4 °C. The resulting pellet was rinsed twice with 4 mL of Nuclei PURE storage buffer (Sigma), and the nuclei were collected by centrifugation at 500*g* for 5 min at 4 °C.

Nuclear Scaffold/Matrix Isolation. Nuclear scaffold isolation was performed as described previously (*29*). Briefly, isolated nuclei were resuspended in 1 mL of digestion buffer (10 mM Tris·HCl, pH 7.4, 20 mM KCl, 0.125 mM spermidine, 0.05 mM spermine, and 1% thiodiglycol) and subjected to digestion by adding 100 units of DNase I and 5 mM $MgCl_2$ and incubating on ice for 30 min. The mixture was then incubated at 37 °C for 10 min after adding $CuSO_4$ to a final concentration of 1 mM. Scaffold proteins were precipitated with an equal volume of 0.4 M $(NH_4)_2SO_4$ prepared in 10 mM Tris·HCl, pH 7.4, and 0.2 mM $MgCl_2$. The precipitate was resuspended in 15 mL of 10 mM Tris·HCl, pH 7.4, containing 0.2 mM $MgCl_2$ and 0.2 M $(NH_4)_2SO_4$ and pelleted at 300*g* at 4 °C for 15 min. The pellet was washed three times with PBS containing 0.1% Triton X-100, 2 mM DTT, and 70 mM NaCl and resuspended in 200 μL of PBS.

Immunofluorescence Confocal Microscopy. Liver was isolated from BalbC mice immediately after decapitation and fixed in 4% paraformaldehyde in PBS at room temperature for 2 h. After three washes in PBS (30 min each), the liver samples were placed in 30% sucrose in PBS overnight at 4 °C. The samples were mounted in O.C.T. medium and processed for cryosectioning using a Leica cryostat (10 μm in thickness) and collected on poly(L-lysine)-coated slides (Sigma). Tissue sections on the slides were treated with cold methanol (20 °C) for 2−3 min. HepG2 cells grown on eight-well Lab-Tek II chamber slides were washed first with serum-free medium and then with PBS. Cells were fixed with 4% paraformaldehyde in PBS for 15 min followed by a PBS rinse and 3 min immersion in cold methanol (−20 °C). Thereafter, both samples were treated similarly. After three washes in TBS (Tris-buffered saline) and a 1 h incubation in TBS containing 0.05% Tween-20 (TBST) and 3% BSA, slides were incubated for 12 h with affinity-purified chicken anti-CBS antibody at 4 °C. Samples were washed with TBST for 3 × 15 min and incubated with Cy5-conjugated F(ab′)2 fragments of donkey anti-chicken antibody in TBST (with 3% BSA) for 1 h at room temperature. Samples were washed two times with TBST followed by 10 min DNA staining with DAPI (4′,6-diamidino-2-phenylindole,1 μg/mL in TBS) to visualize nuclei. After being rinsed with TBS, samples were mounted and examined with an Olympus FV500 confocal laser scanning microscope at the Microscopy Core Facility of the Biotechnology Center. Double-labeled images were collected sequentially with dual excitation (405 nm laser line for DAPI/nucleus and 647 nm line for Cy5-conjugates/CBS) and dual emission (420 nm/690 nm) mode using the FlowView imaging program.

Mass Spectrometric Analysis. Proteins (obtained by immunoprecipitation with anti-SUMO-1 antibody) were separated by SDS−PAGE as described in the figure legends. Parallel lanes of the same sample were separated by PAGE, and half of the gel was immunoblotted with anti-CBS antibody. This membrane was then aligned with the other half of the gel, and slices corresponding to the 63 and 80 kDa bands were excised. In addition, prestained markers served as additional guides for excising bands in the region of the desired molecular mass. Peptides were identified by in-gel tryptic digestion followed by nanoLC-electrospray ionization (ESI) MS/MS on a quadrupole time-of-flight mass spectrometer (QSTAR XL; Applied Biosystems) at the Mass Spectrometry Core Facility of the Redox Biology Center.

RESULTS

CBS Interacts with Sumoylation Pathway Enzymes. A human brain cDNA library was screened using the yeast two-hybrid approach to identify proteins that interact with CBS. The bait plasmid containing the full-length CBS fused in-frame to the DNA binding domain of GAL4 and a human brain cDNA library fused to the GAL4 activator domain were introduced into yeast cells by mating. To increase the stringency of the selection, the yeast strain PJ694A (*24*) containing three reporter genes whose expression is regulated by GAL4, *HIS3*, *LEU2*, and *lacZ*, under the control of separate promoters was employed. Approximately 1.9×10^6 transformants were screened for the expression of the three reporter genes. A total of 36 colonies, representing approximately one-third of the total, were analyzed. DNA sequencing identified proteins in the sumoylation pathway in 14 of them (Table 1) and included the SUMO conjugation enzyme Ubc9, SUMO ligases PIAS1 (protein inhibitor of activated STAT1) and PIAS3, human polycomb protein HPc2, and a RanGTPase binding protein, RanBPM. Interest-

Table 1: Summary of CBS-Interacting Proteins Identified by Yeast Two-Hybrid Analysis

interacting protein	function	no. of independent colonies
PIASI	SUMO ligase	5
PIAS3	SUMO ligase	3
Ubc9	SUMO conjugating enzyme	2
RanBPM	RanGTPase binding protein	3
HPc2	SUMO ligase	4

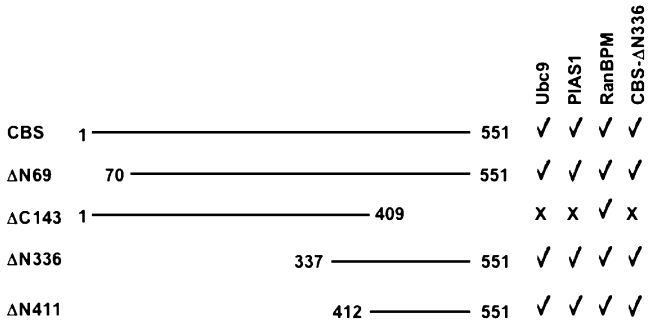

FIGURE 1: Mapping the CBS domain required for interaction with sumoylation proteins. Variants of CBS specified in the figure were fused to the GAL4 DNA binding domain and employed as baits in a yeast two-hybrid assay. The preys were isolated from the initial two-hybrid screen and are fused to the GAL4 activation domain. The regulatory domain of CBS, CBS-ΔN336 domain was identified in the initial screen and served as a useful positive control. The symbols ✓ and x denote the presence and absence of interaction between the bait and prey proteins.

ingly, the C-terminal domain of human CBS, represented in the human brain cDNA library used as prey, and extending from residues 336−551, was also identified in this screen and served inadvertently as a positive control. Cotransformation of prey plasmids with an empty bait plasmid failed to activate expression of reporter genes and served as a negative control.

The C-Terminal Regulatory Domain of CBS Is Required for Interaction with the Sumoylation Machinery. To delineate the region of *CBS* that is required for interaction with proteins in the sumoylation pathway, plasmids containing variously truncated forms of *CBS* were subcloned and fused in-frame with the GAL4 DNA binding domain. These constructs were employed as baits in a yeast two-hybrid assay. The variant CBS-ΔC143 lacking the C-terminal regulatory domain did not interact with Ubc9 or PIAS1 (Figure 1). In contrast, the C-terminal regulatory domain (CBS-ΔN411) itself was capable of interacting with Ubc9, PIAS1, and RanBPM. RanBPM interacted with nonoverlapping domains of CBS, CBS-ΔC143 and CBS-ΔN411, pointing to more than one binding site or the involvement of a third protein present in yeast that mediates this interaction.

CBS Is Sumoylated in Vivo. Standard cell lysis conditions frequently do not allow detection of sumoylated proteins due to the high activity of endogenous isopeptidases that lead to rapid removal of the SUMO modification (*30*). When pig or mouse brain extracts were prepared in the presence of IAA and NEM, which inhibit desumoylases, or were prepared by suspending pulverized brain tissue in hot SDS sample buffer, a band with ∼80 kDa molecular mass was observed that cross-reacted with the anti-CBS antibody (Figure 2A,B). Surprisingly, virtually all the CBS in these extracts appears to be sumoylated rather than a minority fraction, which is typically the case with SUMO target proteins. In these experiments, care was taken to minimize incubation of the control samples (Figure 2A,B) in lysis buffer prior to gel loading. Without this care, the modified CBS band is not observed, explaining why it has not been previously reported in the literature.

Addition of a single SUMO, which has a molecular mass of ∼11 kDa, leads to an apparent increase of ∼20−30 kDa on SDS gels (*31*). The higher molecular weight form of CBS, with an apparent molecular mass of ∼80 kDa (versus 63 kDa for the native protein), was also detected with CBS antibody in immunoprecipitates from brain tissue using SUMO-1 antibody, indicating that CBS is modified by SUMO-1 in vivo (Figure 2). Sumoylation was confirmed by ESI-MS/MS identification of CBS and SUMO-1 peptides in a gel slice containing the 80 kDa form of CBS (not shown). The MS/MS data did not identify a CBS peptide that was sumoylated.

CBS Is Sumoylated in Vitro. To further confirm that CBS is a target for sumoylation, we reconstituted this reaction in vitro (Figure 3). The complete reaction mixture contained purified CBS, SUMO-1, Ubc9, Aos1/Uba2, PIAS1, ATP, an ATP regenerating system, and RRL. Immunoprecipitation using anti-SUMO-1 antibody following a 2−3 h incubation at 37 °C and Western analysis with anti-CBS antibody revealed the presence of two high molecular mass bands corresponding to ∼80 and ∼110 kDa, respectively, and low molecular mass bands that represent degradation products of CBS generated by exposure to RRL (Figure 3, lane 3). Formation of these high molecular mass species required the addition of RRL, indicating that it provides an essential factor needed for efficient sumoylation of CBS (Figure 3, lane 3 versus lane 4). In the absence of RRL, the higher molecular mass bands are very faint whereas the unmodified CBS band is more prominent. This could be explained by the low efficiency of in vitro sumoylation particularly in the absence of RRL. Thus, the unmodified subunits predominate in the native protein and the presence of even a single sumoylated subunit leads to precipitation of the intact tetramer containing the modified (minor band) along with the unmodified (major band) subunits. The signal is specific for CBS and is not observed in the reaction mixture from which this protein is omitted. ESI-MS/MS analysis confirmed SUMO modification of CBS by revealing the presence of SUMO-1 and CBS peptides in both the 80 and 110 kDa bands but did not identify a sumoylated peptide (not shown).

Location of the Sumoylation Site. Two pathogenic mutants of CBS that exhibit significant enzymatic activity when expressed in recombinant form were also tested as substrates in the in vitro sumoylation assay. The linked mutation, P78R/K102N, involves a lysine that resides in a ΨKXE motif and exhibits activity that is only slightly lower than that of wild-type enzyme (Sen and Banerjee, unpublished observation). The second mutation, D444N, is located in the C-terminal regulatory domain and is highly active (*17*). Neither mutation impaired the ability of CBS to be sumoylated under in vitro conditions (Figure 3B).

Next, we examined the effect of mutating eight lysines (K25M, K36M, K39M, K72M, K108M, K137M, K211R, and K398R) on sumoylation of CBS. Mutation of K211, which resides in a ΨKXE motif and is exposed in the structure of the catalytic core (*3*, *32*), resulted in loss of the

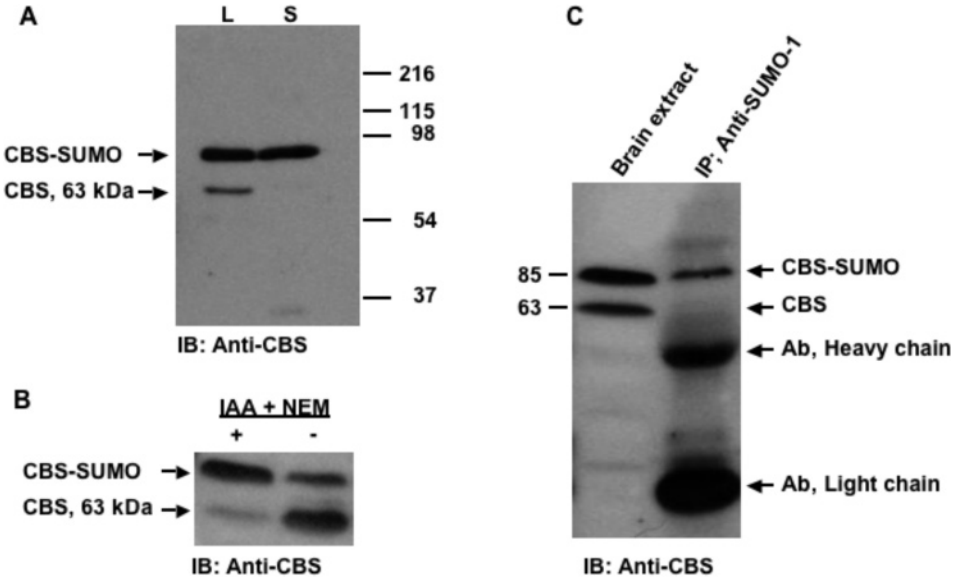

FIGURE 2: CBS is sumoylated in vivo. (A) Pig brain extract was prepared in lysis buffer (L) as described under Materials and Methods or by suspension of frozen powdered brain in hot SDS sample loading buffer (S) and analyzed by immunoblotting with anti-CBS antibody. (B) Pig brain extract was prepared either in the presence (+) or absence (−) of the isopeptidase inhibitors IAA and NEM. (C) Sumoylated CBS was immunoprecipitated with anti-SUMO-1 antibody from pig brain homogenized in buffer containing isopeptidase inhibitors IAA and NEM and detected with anti-CBS antibody as described under Materials and Methods. IP and IB denote immunoprecipitation and immunoblotting, respectively.

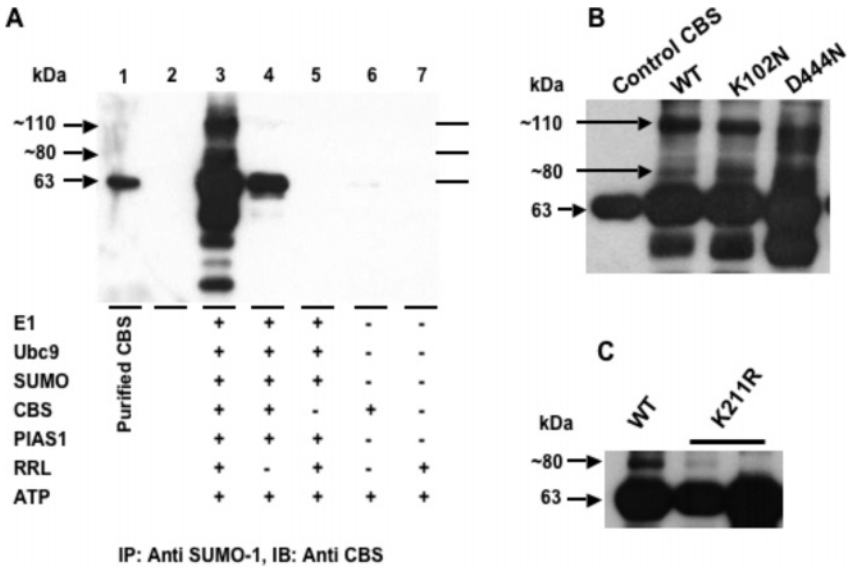

FIGURE 3: CBS is sumoylated in vitro. (A) Sumoylation of wild-type human CBS was analyzed in vitro using purified recombinant enzyme incubated with E1, E2, SUMO-1, ATP, and RRL as described under Materials and Methods. Sumoylation products were immunoprecipitated with anti-SUMO-1 antibody and detected with anti-CBS antibody. (B, C) Comparison of in vitro sumoylation of wild-type and mutant enzymes. The conditions used were identical to those described in lane 3 in (A).

~80 kDa band (Figure 3C). In contrast, mutation of the other lysine residues did not affect sumoylation (data not shown).

CBS Is Present in the Nucleus. Because sumoylation leads to nuclear localization of many target proteins, we tested for the presence of CBS in the nucleus. Two bands, a 63 kDa band that comigrated with purified CBS and an ~80 kDa band, were observed in the nuclei-containing pellet fraction of cell extracts from HepG2 and SH-SY5Y cells (Figure 4A). To corroborate the results from Western blot analysis, we performed immunofluorescence confocal microscopy on HepG2 cells. This analysis confirmed the presence of CBS in the nuclear compartment (Figure 4B).

Nuclei purified from liver and brain showed cross-reactivity to the nuclear marker proteins, lamins A and C (Figure 5A). That the nuclei were free of cytoplasmic contamination was established by the absence of the cytoplasmic protein, methionine synthase, as revealed by Western analysis. Anti-CBS antibody highlighted both the 63 and ~80 kDa bands in purified nuclei (Figure 5A). The presence of unmodified CBS and additional lower molecular mass bands likely result from desumoylation and partial degradation of CBS during the extended nuclear purification protocol.

To further localize CBS in the nucleus, the nuclear scaffold proteins were purified. Western blot analysis demonstrated the presence of the 63 kDa form of CBS (Figure 5B). The absence of the modified form likely resulted from the activity of desumoylases during the lengthy protocol for nuclear subfractionation. The low molecular mass cross-reacting

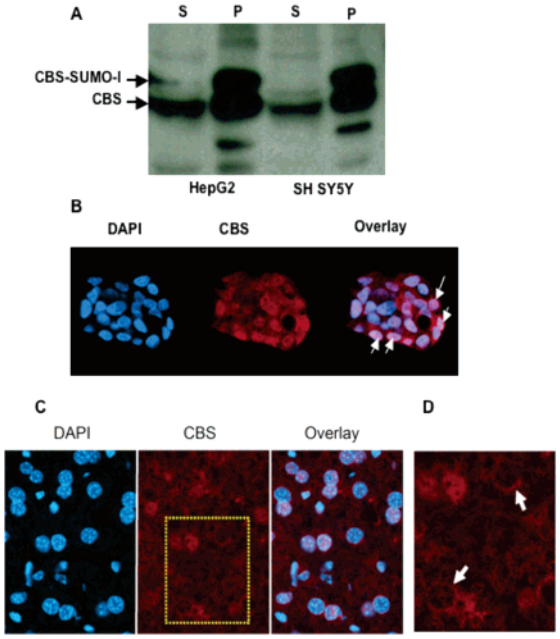

FIGURE 4: Presence of CBS in the nucleus. (A) Cells (HepG2 or SH-SY5Y) were lysed in the presence of 0.1% Triton X-100 for 30 min on ice. Extracts were subjected to centrifugation at 10000*g* for 10 min at 4 °C. Proteins from the soluble supernatant (S) and the nuclei-containing insoluble pellet (P) fractions were separated by SDS−PAGE and detected by anti-CBS antibody. (B) Intracellular localization of CBS detected by immunofluorescence microscopy. HepG2 cells were fixed and stained for nuclei with DAP1 (left column) and labeled with purified anti-CBS antibodies (middle column) as described under Materials and Methods. The overlay in the right column reveals purple nuclei (white arrows) confirming the presence of CBS in that compartment (top panel). The control in which preimmune serum was employed to detect CBS in HepG2 cells showed no staining (not shown). (C) Intracellular localization of CBS in murine liver. Liver slices were fixed and stained for nuclei with DAP1 (left column) and labeled with purified anti-CBS antibodies (middle column) as described under Materials and Methods. The overlay in the right column confirms the presence of CBS in the nucleus. (D) An expanded view of the image outlined by a dotted yellow rectangle in (C) showing localization of CBS around the nuclear envelope (indicated by white arrows) in some cells.

bands observed in the whole nuclear preparation (Figure 5A) were also observed in the nuclear scaffold fraction (Figure 5B).

To complement the in vitro localization of CBS, immunohistochemical localization of the protein was conducted on murine liver sections (Figure 4C). An interesting pattern of localization was seen with respect to the nucleus. While some nuclei stained heavily with anti-CBS antibody, the protein appeared to be concentrated around the nuclear envelope in others (Figure 4D).

DISCUSSION

CBS functions to divert homocysteine from the methionine cycle to cysteine synthesis via the transsulfuration pathway. Mutations in CBS result in hyperhomocysteinemia and affect four major organ systems (*33*). Seizures and psychiatric and behavioral disorders are often observed in patients with CBS deficiency, which is the most frequent abnormality of the central nervous system (*34*, *35*). The molecular basis of the organ-specific pathologies associated with aberrations in CBS is not understood. The human enzyme exhibits complex regulation including intrasteric inhibition by the C-terminal regulatory domain (*7*, *17*), allosteric activation by AdoMet (*4*), and redox regulation that is correlated with reduction of its heme cofactor from the ferric to the ferrous state (*26*). In this study, we have uncovered a previously unknown mode of regulation of CBS, i.e., by sumoylation.

Sumoylation is a posttranslational modification that regulates the function of a growing list of proteins (*23*, *36*). The sequence of reactions for sumoylation of target protein is similar to that for ubiquitination, although the components are distinct. In some cases, SUMO and ubiquitin compete for the same target lysine residue, and sumoylation protects the protein from degradation (*37*−*39*).

In this study, a yeast two-hybrid analysis identified multiple components of the sumoylation pathway as interacting partners of CBS (Table 1). In addition to the E2 conjugating enzyme, Ubc9, we isolated three E3 SUMO ligases (PIAS1, PIAS3, and HPc2), which function in the cell to increase the specificity and efficiency of sumoylation. It is possible that isolation of multiple SUMO ligases was a consequence of the formation of a multiprotein complex involving an E3 prey with an E2 present in yeast cells, which in turn interacts with the bait, CBS.

The relevance of the yeast two-hybrid results was established by demonstrating that CBS is sumoylated under both in vitro and in vivo conditions (Figures 2 and 3). Sumoylation is a dynamic process that is reversed by desumoylases. This makes it difficult to detect sumoylated proteins in cell extracts due to rapid cleavage of the isopeptide bond between SUMO and the target protein (*30*). This could explain why sumoylation of CBS has been missed previously. However, this modification of CBS was clearly observed when care was taken to minimize the time between cell lysis and gel loading, and cysteine protease inhibitors that are commonly employed to inhibit desumoylases or boiling lysis buffer were used (Figure 2). It is interesting to note that sumoylation of several proteins has been discovered by the yeast two-hybrid analysis (*40*−*43*). Surprisingly, CBS appeared to be predominantly in the sumoylated form in the brain in contrast to most sumoylated proteins in which only a fraction appears to be modified. Sumoylation of CBS was supported by immunoprecipitation studies with anti-SUMO-1 antibody followed by Western blot detection with anti-CBS antibody (Figure 2). The presence of modified CBS was confirmed by MS/MS identification of both SUMO-1 and CBS peptides associated with this band in the gel (not shown).

Deletion analysis localized the C-terminal regulatory domain of CBS as being important for interactions with the sumoylation machinery (Figure 1). The CBS-ΔC143 variant missing the regulatory domain did not interact with two of the three sumoylation enzymes that were tested and was not a substrate for sumoylation in the in vitro assay (not shown). We cannot explain its apparent interaction with RamBPM. Consistent with the importance of the regulatory domain in interactions with the sumoylation machinery, the C-terminal domain by itself was found to interact with all three sumoylation machinery partners that were tested. As a positive control, the C-terminal domain CBS-ΔN336, which had been identified as an interacting partner of full-length CBS in the initial yeast two-hybrid screen, was employed as prey and interacted with all the bait variants that contained the C-terminal domain, consistent with the role of this

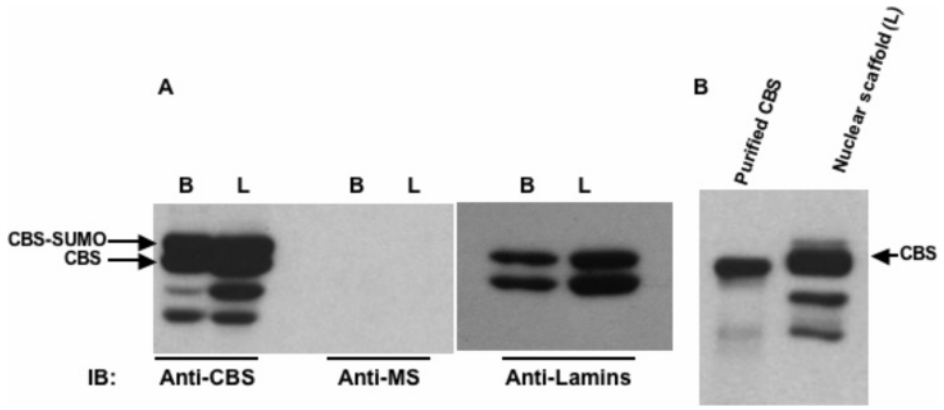

FIGURE 5: Nuclear scaffold localization of CBS. (A) Nuclear lysate was separated on SDS−PAGE and analyzed by Western blotting using anti-CBS antibody. Both the unmodified and SUMO-modified CBS forms were detected. Anti-methionine synthase antibody and anti-lamin antibody were employed as negative and positive controls to detect for a cytoplasmic- and a nuclear-specific protein, respectively. B and L refer to brain and liver extracts, respectively. (B) Nuclei were subjected to lysis, and the scaffold proteins were isolated as described under Materials and Methods. CBS was detected using the anti-CBS antibody.

domain in oligomerization of the protein (*6*). These results support the importance of the regulatory domain in interactions with components of the sumoylation machinery. They do not, however, localize the sumoylation site to the regulatory domain.

While a single high molecular mass band that cross-reacts with anti-CBS antibody is observed under in vivo conditions, two bands are seen in the in vitro sumoylation assay (Figure 3). This indicates that CBS is modified by more than 1 equiv of SUMO under these conditions and could result from attachment of two SUMO molecules at distinct acceptor lysines. The alternative, i.e., formation of a SUMO chain, is less likely since this has not been observed with SUMO-1. CBS contains three lysines (K102, K211, and K398) that are embedded in a canonical ΨKXE sumoylation motif. Of these, K102 has been reported to be the site of a pathogenic mutation and is inherently linked to a second mutation at P78 (*15*). However, the P78R/K102N mutant of CBS is sumoylated in vitro (Figure 3B), eliminating this lysine as the site for modification. In addition, sumoylation of the D444N mutant, which is seen in several homocystinuric patients but exhibits robust activity when expressed as a recombinant protein (*17*), is also a substrate for sumoylation under in vitro conditions (Figure 3B). In contrast, mutation of K211 led to loss of sumoylation, indicating that this lysine residue may represent the site of modification.

Sumoylation can influence nucleocytoplasmic transport, and a majority of sumoylated proteins are located in the nucleus (*23*). The observed sumoylation of CBS prompted examination of whether this protein has a nuclear residence in addition to its known cytoplasmic one. The presence of CBS was observed in isolated nuclei derived from both brain and liver cells and was further localized to the nuclear scaffold (Figure 5). Immunofluorescence detection of CBS in HepG2 cells (Figure 4B) and in murine liver slices (Figure 4C) showed that it is found in both the nuclear and cytoplasmic compartments. Sumoylation is a dynamic process, and the signals that govern the localization of CBS to the nuclear compartment are presently unknown. The histochemical studies on liver sections reveal the concentration of CBS around the nuclear envelope in some cells and inside the nucleus in others (Figure 4C). A subcellular localization study of CBS in developing monkey brain reported that the crude nuclear fraction contained ~10% the enzyme activity found in the soluble fraction (*44*). However, the levels were sufficiently low that the possibility of contaminating soluble fraction contributing to CBS activity seen in the crude nuclear fraction could not be ruled out. Our studies establish the nuclear residence of mammalian CBS.

Functional categories of known SUMO targets include proteins involved in stress response, chromatin stability−gene silencing, DNA repair and genome stability, transcription, translation, and RNA metabolism as well as a variety of metabolic enzymes including superoxide dismutase, isoform b of adenylate kinase, and ornithine aminotransferase (*45*, *46*). The physiological significance of sumoylation and localization of CBS in the nucleus is an important issue that is not addressed in the present study. While it is possible that sumoylation regulates the activity of CBS, its location in the nucleus raises the possibility of an alternative and as yet unknown role for this protein in this compartment. CBS is a heme protein that binds CO with consequent inhibition of activity, which raises the possibility that it may function as a CO sensor (*47*). We speculate that sumoylated CBS may modulate downstream functions in the nucleus in response to changes in redox or CO binding. It is interesting to note that a nuclear receptor, E75, was recently demonstrated to be a heme protein and is responsive to CO and NO (*48*).

It is also interesting to note that both the transsulfuration pathway and sumoylation play protective roles under stress conditions. Thus, the flux of homocysteine through the transsulfuration pathway increases when cells are exposed to oxidative stress and leads to increased synthesis of the major cellular antioxidant, glutathione (*28*, *49*). A global increase in sumoylation and of specific SUMO targets has been reported in response to stress elicited by heat shock, hydrogen peroxide, ethanol, and heavy metal treatments (*45*, *50*−*52*). Sumoylation of CBS is prominent in the brain (Figure 2) and raises the possibility that this may be relevant to the etiology of central nervous system disorders associated with deficiency of this enzyme. CBS was identified as an interacting partner for Huntingtin protein (*18*). Accumulation of mutant Huntingtin containing an expanded polyglutamine tract is associated with the neurodegenerative disease, and relocalization of a pathogenic fragment of the protein from the cytoplasm to intranuclear inclusions is observed (*53*).

Interestingly, SUMO modification of a pathogenic fragment of Huntingtin diminishes aggregate formation and competes for ubiquitination at the same site (*22*). The significance of the interaction between CBS and Huntingtin and the possible relevance to Huntington's disease pathology are not known.

In summary, we have identified a modification of human CBS that was previously unknown, i.e., sumoylation, and demonstrated that the protein resides in both the cytoplasmic and nuclear compartments. It is likely that the lability of the SUMO modification of CBS in cell extracts thwarted its earlier identification. The discovery of this modification raises the possibility that a subclass of CBS mutations may be impaired in their interactions with proteins in the sumoylation machinery. Our discovery of a new residence for CBS opens the doors to elucidating its interactions with other proteins and its role in this compartment that could be relevant to the molecular basis of the clinical manifestations of its deficiency. The physiological relevance of this modification and an alternative role for CBS in the nucleus are important questions that will be addressed in the future.

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