Ankyrin Repeat and Suppressors of Cytokine Signaling Box Protein Asb-9 Targets Creatine Kinase B for Degradation*

The suppressors of cytokine signaling (SOCS) proteins inhibit cytokine action by direct interaction with Janus kinases or activated cytokine receptors. In addition to the N-terminal and Src homology 2 domains that mediate these interactions, SOCS proteins contain a C-terminal SOCS box. DNA database searches have identified a number of other protein families that possess a SOCS box, of which the ankyrin repeat and SOCS box-containing (Asb) proteins constitute the largest. Although it is known that the SOCS proteins are involved in the negative regulation of cytokine signaling, the biological and biochemical functions of the Asbs are largely undefined. Using a proteomics approach, we demonstrate that creatine kinase B (CKB) interacts with Asb-9 in a specific, SOCS box-independent manner. This interaction decreases the polyubiquitylation of CKB and decreases total CKB levels within the cell. The targeting of CKB for degradation by Asb-9 was primarily SOCS box-dependent and suggests that Asb-9 acts as a specific ubiquitin ligase regulating levels of this evolutionarily conserved enzyme.
The Asbs have been implicated in different biological processes; Asb-2 may regulate myeloid cell proliferation and/or differentiation (16, 17), Asb-5 plays a possible role in the initiation of arteriogenesis (18), and Asb-11 may regulate the proliferation and differentiation of the developing nervous system (19), whereas Asb-15 has been reported to regulate muscle growth by acting as a negative regulator of proliferating muscle cells and by increasing the rate of protein synthesis in differentiated myoblasts (20, 21). Asb-8 has been implicated in cancer, with Asb-8 expression undetectable in normal adult lung tissue but present in several lung carcinoma cell lines. Transfection of a possible dominant negative form of Asb-8 (human Asb-8 cDNA lacking the SOCS box) suppressed the growth of lung adenocarcinoma cells in vitro, implying an association of Asb-8 with the development of lung cancer (22). The function of Asb-1 was studied by utilizing genetically modified mice. Although Asb-1 knock-out mice displayed some testicular anomalies, it was concluded that deletion and overexpression of Asb-1 had no obvious effect on mouse development, thus suggesting a possible redundancy between Asb proteins (23).

Recent studies propose that the Asbs perform an analogous role to the SOCS proteins, regulating various signaling pathways via an interaction between the SOCS box motif and the Elongin B/C complex to initiate ubiquitylation and proteasomal degradation of proteins bound to the ankyrin repeat region. One study reported that Asb-2 may target regulators of hematopoiesis for degradation by assembling into an ECS-type E3 ubiquitin ligase with the Elongin B/C complex, Cullin-5, and Rbx-1 (24). In a separate study, TNF-R2-mediated cellular responses to TNF-α were negatively regulated by Asb-3. Downregulation of Asb-3 by RNA interference led to an accumulation of TNF-R2 and TNF-R2-associated cytotoxicity (25). Finally, Asb-6 was found to interact with the adaptor protein APS (adapter protein with pleckstrin homology and SH2 domain), which couples the insulin receptor to components of a glucose transport pathway. Following prolonged insulin stimulation, APS was degraded when Asb-6 was overexpressed (26).

Creatine kinase, an evolutionarily conserved enzyme, is critical for the maintenance and regulation of cellular energy stores in tissues with high and rapidly changing energy demands, such as skeletal and cardiac muscle and the brain. In mammals, three cytosolic (CKM, CKB, and CKMB) and two mitochondrial (CKMt1 and CKMt2) isoforms of creatine kinase are expressed. CKM is muscle-specific, CKMb, a heterodimer of both muscle and brain subunits, is predominantly expressed in heart, and the two mitochondrial creatine kinase isoforms, ubiquitous CKMt1 and sarcomeric CKMt2, are located in the mitochondrial intermembrane space and are often co-expressed with the cytoplasmic creatine kinases. The brain type cytosolic enzyme of creatine kinase, CKB, plays a major role in cellular energy metabolism of non-muscle cells. CKB is expressed in a range of tissues, mainly in the brain and retina, but also in the uterus, placenta, kidney, and testes. There is ample evidence that the CK system is linked with brain and muscle function (reviewed in Ref. 27). A number of neurological and muscular diseases display perturbations in CK activity and creatine metabolism, although the causal relationships of many are not known. A role for CKB in brain function is further supported by altered behavioral patterns observed in CKB knock-out mice (28).

Overexpression of CKB has been observed in a number of tumors, including neuroblastoma, small cell lung carcinoma, colon and rectal adenocarcinoma, and breast and prostate carcinoma as well as some tumor cell lines (reviewed in Refs. 27 and 29). Elevated CKB expression was also reported in B-lineage cells from patients with acute lymphoblastic leukemia (30). Furthermore, CKB is induced by the adenovirus E1a oncogene (31). Conversely, wild-type p53 repressed the CKB promoter (32). In fact, many human small cell lung carcinomas, which exhibit elevated CKB expression, contain mutations in p53 alleles (reviewed in Ref. 27).

Since SOCS box-containing proteins target specific proteins for degradation via a SOCS box-dependent manner, we reasoned that the key to elucidating the function of the Asb protein family is to study the proteins with which they interact. Here, we report the identification of CKB as a specific binding partner of Asb-9 with in vitro and in vivo confirmation of the interaction in primary cells. We show that the interaction leads to CKB ubiquitylation and degradation in a SOCS box-dependent manner, suggesting that Asb-9 acts as a specific ubiquitin ligase regulating CKB abundance.

**EXPERIMENTAL PROCEDURES**

**Northern Hybridization**—Tissues were dissected from 8-week-old C57BL/6 mice and immediately snap frozen in liquid nitrogen. Total RNA was extracted from tissues using TRIzol reagent according to the manufacturer’s instructions (Invitrogen). Northern blots were performed after electrophoresis, as described (33). For Northern blot hybridization, the entire coding region of the mouse Asb-9 cDNA was used. The membrane was stripped and rehybridized with a 1.2-kbp PsI fragment of the chicken glyceraldehyde-3-phosphate dehydrogenase cdNA to control for RNA loading and integrity.

**Expression of Asb-9 and CKB in 293T Cells**—Total cellular RNA was isolated using TRIzol reagent (Invitrogen) as per the manufacturer’s instructions. First strand cDNA synthesis was performed using Superscript III RNase H− reverse transcriptase (Invitrogen). Forward (F) and reverse (R) oligonucleotides specific for the sequence of human Asb-9 and CKB were designed as follows: Asb-9, 5′-GAGTCAGGACCGACCAGTG-CGTG-3′ (F) and 5′-CAGTTCCTGCAAGCTATTGTT-3′ (R); CKB, 5′-CGGTATCCTGACCAATGACA-3′ (F) and 5′-GGGGTGAACACCTCTTCCATG-3′ (R). PCR conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 35 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s.

**Expression Vectors for Transient Transfections**—The cDNAs encoding Asb-1 to Asb-12, Asb-14, Asb-15, SOCS-3, and WSB-1 were obtained as described (2, 4, 34). Constructs encoding these proteins, with or without the SOCS box, with an N-terminal FLAG epitope tag (DYKDDDDK) were generated by PCR to restriction enzyme sites at both the N and C termini and were subcloned into the mammalian expression vector pEF-FLAG-I.

**Transfection of 293T Cells**—Human embryonic kidney 293T cells were plated at a density of 8 × 10⁶ cells/Nunc175-cm² tissue culture flasks (Nalge Nunc International) or in 6-well...
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Costar plates (Corning Glass) at 0.5 × 10^6 cells/well and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum (HyClone Laboratories). Cells were incubated overnight at 37 °C in a humidified atmosphere of 10% CO₂ in air and transfected with a maximum of 2.5 μg of pEF-FLAG-I expression vector containing the cDNA of interest, using FuGene transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. Where indicated, the proteasomal inhibitor PS341 was used at a concentration of 10 nm diluted in Me₂SO. Cells were treated with PS341 for 24 h.

Transfection of HeLa Cells with Asb-9—Human epithelial cervical carcinoma HeLa cells were plated in 6-well Costar plates as described for the 293T cells. HeLa cells were transfected with 0–2.5 μg of empty vector and pEF-FLAG-Asb-9 or pEF-FLAG-Asb-9 lacking the SOCS box (/ΔSB) using the Lipofectamine transfection reagent (Invitrogen) according to the manufacturer’s instructions.

Constructs for Stable Cell Lines—HA-ubiquitin N terminus FLAG was amplified from pcDNA5 FRT TO HA-ubiquitin as described elsewhere (35) with oligonucleotides 5’-GCTGATGC-GCCGCCGCGCTTAGACGAGTACGGCCGGCCGGATCC-CTTGTCACTCGTCCTTTGTAGCTAATTGCCCCACC–TCTGAG-3’ and 5’-GCCGGTACACCCATGGAACGATC-CCCTATTAGCCTCC-3’, digested with KpnI and NotI, and inserted into pcDNA5 FRT TO digested with KpnI and NotI to create pcDNA5 FRT TO HA-ubiquitin N-FLAG. This vector was digested with Ascl, and the Asb-9 and Asb-9/ΔSB inserts were cloned in with Ascl and MluI from pEF-Asb-9 and pEF-Asb-9/ΔSB. All constructs were verified by sequencing throughout the complete coding sequence.

Generation of Stable Cell Lines—Stable cell lines were established by transiently transfecting the Flp-In™ T-REx™ 293 cell line (Invitrogen) with pcDNA5 FRT TO constructs (Invitrogen) with the recommended amount of pOG44. 24 h after transfection, cells were split into 15-cm tissue culture plates and selected with 500 μg/ml hygromycin (Invitrogen) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen). After 1 week, individual colonies were picked and then expanded and tested for doxycyclin (Sigma) resistance against full-length Asb-9 or a mouse monoclonal antibody, whereas endogenous CKB was detected by antibody raised against a peptide mapping at the amino terminus of CKB (sc-15157; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a rabbit anti-CKB polyclonal antibody (40), whereas endogenous CKB was detected by antibody raised against a peptide mapping at the amino terminus of CKB (sc-15157; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a rabbit anti-CKB polyclonal antibody (40), whereas endogenous CKB was detected by antibody raised against a peptide mapping at the amino terminus of CKB.
at 1:200 and rabbit anti-CKB (Fitzgerald) at 1:1000. Secondary antibodies used were Alexa Fluor 488 goat anti-rabbit polyclonal (Molecular Probes) at 1:1000 and Cy5 goat anti-rat (Jackson Laboratories) at 1:500.

**Generation of MYC-tagged CKB and Detection of Ubiquitylated Protein**—A DNA clone encoding creatine kinase B in a pCMV-SPORT6 vector was purchased from the I.M.A.G.E Consortium (supplied by the Medical Research Council GeneService) (ID 4225384). Oligonucleotides 5′-ACGTTGCGCGCCAGCCCT-TTCTCACCAGCCCTATACG-3′ and 5′-ACGTACCGCTTGGGGCGCATGAGGTAC-3′ were used to amplify the CKB coding sequence with in frame Ascl and MluI sites at the 5′ and 3′ ends. The PCR-generated fragment was digested with Ascl and MluI and then subcloned into pEF-MYC-1 to generate a CKB construct with a C-terminal MYC (DQKLISEEDL) tag. The MYC-tagged CKB plasmid, an HA-tagged ubiquitin plasmid, and a FLAG-tagged Asb-9 plasmid or its deleted SOCS box form were transfected into 293T cells as described above. Clarified cell extracts were immunoprecipitated with anti-MYC antibody, and ubiquitylated protein was detected by anti-HA antibody (Roche Applied Science).

**Pulse-Chase Analysis of Creatine Kinase B**—293T cells were transfected with the MYC-tagged CKB plasmid and either the pEFBOS expression vector, FLAG-Asb-9, or FLAG-Asb-9/ΔSB plasmids as described above. At 48 h post-transfection, cells were rinsed with methionine-free Dulbecco’s modified Eagle’s medium supplemented with 0.1% bovine serum albumin (AlbuMAX I 10% solution; Invitrogen). Cells were radiolabeled for 5 min with 0.1% (v/v) l-[35]S-methionine and pulse-labeled for 0.1M. To assess the purity of GST-Asb-9 in Freund’s incomplete adjuvant. A final antigen challenge with 30 μg of His-Asb-9 in Freund’s incomplete adjuvant was administered 3 days before spleens were removed. Spleen cells were fused with the SP2/O mouse myeloma cell line. Hybridomas, for which anti-Asb-9 reactivity was detected, were cloned by limiting dilution and supernatants from hybridoma clones were screened by enzyme-linked immunosorbent assay for their ability to recognize both GST-Asb-9 and His-Asb-9. These supernatants were rescreened by enzyme-linked immunosorbent assay to test for their ability to bind to protein G in order to select for supernatants containing IgG antibodies, which are most suitable for their downstream applications, namely immunoprecipitation and Western blotting.

To purify anti-Asb-9 antibodies, hybridoma supernatant was passed through a protein G column (Amersham Biosciences), and antibody was eluted with 0.1 M glycine buffer (pH 2.7). The eluted antibody solution was neutralized with the addition of Tris, pH 8, to a final concentration of 0.1 M. To assess the purity of the antibodies, ~0.5 μg of antibody was separated by SDS-PAGE under both reducing and nonreducing conditions. The clone 5D3 was used to detect endogenous Asb-9 where indicated in this study.

**RESULTS**

Expression of Asb-9 in Vivo—In the adult mouse, Asb-9 mRNA expression was detected predominantly in the testes and kidney, with low expression observed in the heart and liver (Fig. 1). Asb-9 expression was undetectable in all other tissues examined. Since Asb-9 mRNA was normally expressed in the kidney, we utilized the human embryonic kidney 293T cell line to examine possible interactions of endogenous proteins with Asb-9 in addition to the standard overexpression studies.

Identification of CKB as an Asb-9-specific Interacting Protein—Proteins that associate with Asb-9 were purified from 293T cells expressing FLAG-tagged Asb-9 using anti-FLAG M2 antibody (Roche Applied Science). The FLAG-tagged Asb-9 plasmid was expressed in addition to the standard overexpression studies.

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Dynamics) and quantified using ImageQuant software (version 5.0). The fraction of 35S-labeled MYC-CKB remaining at each time point was then calculated to allow the half-life of the protein to be estimated.

**Generation of Anti-Asb-9 Monoclonal Antibodies**—For the generation of anti-Asb-9 monoclonal antibodies, BALB/c mice were immunized with glutathione S-transferase (GST)-tagged Asb-9 and His-tagged Asb-9 recombinant proteins. To produce GST-Asb-9 protein, the cDNA of murine Asb-9 was subcloned into a modified pGEX-2T vector (Amersham Biosciences) as a GST fusion protein. The GST-Asb-9 was expressed in *Escherichia coli* strain NM522 cells and purified according to the manufacturer’s instructions with minor modifications. Briefly, the isopropyl 1-thio-β-D-galactopyranoside-induced *E. coli* pellets were lysed on ice for 1 h in 20 ml of lysis buffer (1% (v/v) Triton X-100, 0.2 mg/ml lysozyme (Sigma), 1 mM phenylmethylsulfonyl fluoride, 30 μM/ml DNase I (Roche Applied Science) in PBS. Lysates were subjected to centrifugation at 20,000 × g for 15 min. The bacterial lysate was incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) for 1 h at 4 °C. Beads were washed with 1% (v/v) Triton X-100 in PBS and then 1% (v/v) Triton X-100 in 50 mM Tris-HCl pH 8.0, 150 mM NaCl (TBS). Bound GST-Asb-9 was eluted in 10 1-ml fractions with 50 mM glutathione in 1% (v/v) Triton X-100 in TBS. Fractions containing the GST fusion protein were pooled and dialyzed against PBS overnight at 4 °C. Murine Asb-9 was also cloned into a pET15b vector (Novagen) and expressed as a His8-tagged protein in BL21 DE3 pLysS *E. coli* (Stratagene). The His-tagged Asb-9 protein was expressed predominantly as an insoluble protein and purified using Ni2+-nitritoltriacetic acid resin (GILAGEN) under denaturing conditions according to the manufacturer’s instructions. Fractions containing the His-tagged protein were eluted in 6 M guanidine HCl, 100 mM NaH2PO4, 10 mM Tris-HCl, pH 4.5, were pooled and purified further by reversed phase high pressure liquid chromatography on a 100 × 7.5-mm inner diameter Vydac C4 column with a 60-min linear gradient of 0–100% acetonitrile in 0.085% (v/v) trifluoroacetic acid, lyophilized, and reconstituted in Milli-Q water.

BALB/c mice were immunized with 30 μg of GST-Asb-9 in Freund’s complete adjuvant and were then boosted with 30 μg of GST-Asb-9 in Freund’s incomplete adjuvant. A final antigen challenge with 30 μg of His-Asb-9 in Freund’s incomplete adjuvant was administered 3 days before spleens were removed. Spleen cells were fused with the SP2/O mouse myeloma cell line. The hybridomas were screened by enzyme-linked immunosorbent assay for their ability to recognize both GST-Asb-9 and His-Asb-9. These supernatants were rescreened by enzyme-linked immunosorbent assay to test for their ability to bind to protein G in order to select for supernatants containing IgG antibodies, which are most suitable for their downstream applications, namely immunoprecipitation and Western blotting.

To purify anti-Asb-9 antibodies, hybridoma supernatant was passed through a protein G column (Amersham Biosciences), and antibody was eluted with 0.1 M glycine buffer (pH 2.7). The eluted antibody solution was neutralized with the addition of Tris, pH 8, to a final concentration of 0.1 M. To assess the purity of the antibodies, ~0.5 μg of antibody was separated by SDS-PAGE under both reducing and nonreducing conditions. The clone 5D3 was used to detect endogenous Asb-9 where indicated in this study.
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These proteins were excised from the gel, digested with trypsin in situ, and identified by mass spectrometry (Table 1). Consistent with experiments of other SOCS box-containing proteins, Elongins B and C (18 and 15 kDa, respectively) and Cullin-5 (90 kDa), co-immunoprecipitated with Asb-9. In contrast, creatine kinase B had not been previously identified in SOCS protein immunoprecipitation experiments and therefore interacted with Asb-9 in an apparently specific manner.

Expression of Asb-9 and CKB in 293T Cells—The expression of endogenous Asb-9 and CKB in 293T cells at the mRNA and protein level was examined by reverse transcription-PCR and Western blot. Asb-9 was expressed at low levels in 293T cells and was detected by reverse transcription-PCR and by Western blot using a rabbit polyclonal antibody raised against Asb-9. In contrast, creatine kinase B mRNA and protein was abundant in 293T cells (Fig. 3). As a consequence of the low basal expression of Asb-9, it was overexpressed for further analysis of the Asb-9-CKB interaction in 293T cells.

Specificity of Asb-9-CKB Interaction—The specificity of the Asb-9-CKB interaction was further examined by testing the interaction of CKB with nearly all of the known Asb proteins as well as SOCS-3 and WSB-1. The FLAG-tagged Asb proteins were immunoprecipitated, and association with endogenous CKB was detected by Western blot with anti-CKB antibody. CKB was only detected in immunoprecipitations from cells transfected with Asb-9 and none of the other Asbs, suggesting that the CKB interaction was highly specific to Asb-9 (Fig. 4, A and B). Unsurprisingly, less related proteins, such as SSB-2, WSB-2, and the ankyrin repeat proteins Gankyrin and Harp (data not shown), did not interact with CKB.

SOCS Box-dependent and -independent Interactions—In order to explore the basis of the Asb-9 and CKB interaction, full-length FLAG-Asb-9 or Asb-9 lacking the SOCS box (Asb-9/ΔSOCS) was transiently expressed in 293T cells, and the interaction with CKB was examined. As shown in Fig. 5A, both Asb-9 and Asb-9/ΔSOCS readily interacted with endogenous CKB, suggesting that the binding of the putative substrate CKB to Asb-9 occurs independently of the SOCS box. As expected, however, the SOCS box was critical for interactions with Elongins B and C (Fig. 5C) and Cullin-5 (Fig. 5D) (Fig. 2) (6, 10).

Asb-9 Targets CKB for Degradation in a SOCS Box-dependent Manner—To examine the consequences of the interaction between Asb-9 and CKB, 293T cells were transfected with increasing concentrations (0–2.5 μg) of FLAG-tagged Asb-9 or FLAG-tagged Asb-9/ΔSOCS constructs. Total cell lysates were analyzed by Western blot with an anti-CKB antibody. As the concentration of transfected Asb-9 increased (Fig. 6A, top left panel), levels of endogenous CKB decreased, suggesting that Asb-9 may play a role in regulating the levels of CKB within the cell. In contrast, the levels of endogenous CKB were unaffected in cells expressing Asb-9/ΔSOCS (Fig. 6A, top right panel), indicating that, whereas the binding of CKB by Asb-9 occurs via the ankyrin repeats, the effect on CKB protein levels is dependent on an intact SOCS box. The consequence of Asb-9 overexpression on endogenous CKB levels was also examined in the HeLa cell line. Similar to the 293T experiments, overexpression of Asb-9 resulted in SOCS box-dependent reduction in cellular CKB protein (Fig. 6B).

A similar but more complete degradation was observed using isogenic cell lines that stably and inducibly express Asb-9 or Asb-9/ΔSOCS. Upon induction of full-length Asb-9 by doxycyclin,
no endogenous CKB could be detected via Western blot (Fig. 6C). This was also observed in several independent cell lines (data not shown). Consistent with the transient transfection results, CKB levels remained unchanged when Asb-9/ΔSB was induced. The reduction of the CKB protein was greater in the stable lines compared with the transient expression system, probably because all cells in the inducible stable cell lines expressed the construct, whereas transient transfection could only target a fraction of the total number of cells. A similar complete degradation of a target protein by a RING finger containing E3 ligase has been previously observed using this inducible system (41).

By immunofluorescence and confocal microscopy, the localization of CKB and various FLAG-tagged Asb and SOCS proteins in 293T cells was examined (Fig. 7). CKB was expressed in the cytoplasm of 293T cells and was easily visualized using an anti-CKB antibody (Fig. 7, top row). Overexpressed FLAG-tagged proteins were detected using an anti-FLAG antibody. CKB shared a cytoplasmic location with Asb-9/ΔSB as well as FLAG-tagged Asb-3 and SOCS-3 proteins in 293T cells. Entirely consistent with results presented in Fig. 6, endogenous CKB was undetectable in cells that expressed FLAG-Asb-9 (Fig. 7, second row), whereas levels and location of CKB were unaffected by any of the other SOCS box-containing proteins tested (Fig. 7, rows 3–5). This highlights the reproducibility of this interaction at the single cell level.

To assess further the effect of Asb-9 on CKB degradation, the turnover of the CKB protein was determined via pulse-chase analysis. 293T cells were co-transfected with a MYC-tagged CKB plasmid and either a pEFBOS vector control, FLAG-tagged Asb-9, or FLAG-tagged Asb-9/ΔSB plasmids. Transfected cells were pulse-labeled with [35S]methionine and then chased for various time periods in normal culture medium containing unlabeled methionine (Fig. 8). MYC-tagged CKB was immunoprecipitated with anti-MYC antibody, and labeled proteins were visualized using a PhosphorImager. As before, turnover of CKB was accelerated by co-expression of Asb-9, and the SOCS box was required for this effect, since CKB half-life was similar in the presence or absence of Asb-9/ΔSB (Fig. 8, A and B). Finally, treatment with the proteasomal inhibitor PS341 prolonged CKB half-life (Fig. 8B). These results indicate that Asb-9 promotes the degradation of CKB and that the degradation is SOCS box-dependent and is mediated by the proteasome.

Asb-9 Induces SOCS Box-dependent Ubiquitylation of CKB—SOCS proteins have been reported to induce proteasome-dependent degradation of their target proteins (42, 43). To investigate whether Asb-9-induced reduction of CKB levels was due to SOCS box-mediated ubiquitylation of CKB, we co-expressed MYC-CKB with full-length Asb-9 or Asb-9/ΔSB as well as HA-
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**FIGURE 6.** A–C, SOCS-box dependent degradation of CKB. A, 293T cells were co-transfected with increasing concentrations (0–2.5 μg) of FLAG-tagged Asb-9 or FLAG-tagged Asb-9/ΔSB constructs and decreasing concentrations of pEFBOS vector to ensure that a total of 2.5 μg of DNA was used per transfection. Total cell lysate was immunoblotted with an anti-CKB antibody (upper panels). Expression of FLAG-tagged protein was determined by an anti-FLAG Western blot (WB; middle panels). Actin levels were also examined in the presence of increasing concentrations of Asb-9 to ensure that Asb-9 specifically regulates CKB degradation (lower panels). B, HeLa cells were transfected with 0–2.5 μg of FLAG-Asb-9 or FLAG-Asb-9/ΔSB. The pEFBOS vector was also transfected to ensure that a total of 2.5 μg of DNA was used per transfection. Endogenous CKB was detected using an anti-CKB antibody (upper panels). The expression of FLAG-tagged Asb-9 or Asb-9/ΔSB was confirmed by an anti-FLAG Western blot (lower panels). C, stable 293 cell lines were uninduced (lane 1) or induced to express FLAG-Asb-9 (lane 2) and FLAG-Asb-9/ΔSB (lane 3) by the addition of doxycyclin. Total cell lysate was immunoblotted with anti-CKB (upper panel). Expression of FLAG-tagged protein was determined by an anti-FLAG Western blot (middle panel). The membrane was reprobed with anti-Hsp70 as a loading control.

ubiquitin. As shown in Fig. 9A, little basal polyubiquitylation of CKB was observed (lane 3); however, co-expression of Asb-9 (lane 4) but not Asb-9/ΔSB (lane 5) resulted in enhanced polyubiquitylation of CKB. Upon treatment of cultures with the proteasomal inhibitor PS341, a markedly increased level of polyubiquitylated CKB was observed (lanes 6–8). Importantly, the ubiquitylation of CKB was substantially enhanced on co-expression with Asb-9 (lane 7), but to a reduced extent with overexpressed Asb-9/ΔSB, supporting our previous observations. To confirm that the ubiquitylated protein smears observed in the Asb-9 co-transfections (lanes 4 and 7) were due to modification of the MYC-tagged CKB protein by ubiquitin, the blot was stripped and reprobed with anti-MYC (Fig. 9B). The reprobe shows a strong band that most likely corresponds to a monoubiquitylated form of CKB and was predominant when Asb-9 was co-expressed. As expected, the band appeared more intense following treatment with PS341. Unmodified MYC-CKB protein was easily detected, and no obvious differences in levels were observed in the untreated versus PS341-treated samples. This may be attributed to the immunoprecipitation process, since an increase in MYC-tagged CKB protein levels upon PS341 treatment was observed when total cell lysate was examined via immunoblot with anti-MYC (D).

**DISCUSSION**

Using a proteomic approach, we identified CKB as a protein capable of specifically interacting with Asb-9. CKB is a key cytosolic enzyme in cell energy metabolism (reviewed in Ref. 44) reversibly catalyzing the ATP-dependent phosphorylation of creatine and, hence, provides an ATP buffering system for tissues requiring large amounts of energy. Subsequent experiments established that the interaction was unique to Asb-9 and that the ankyrin repeat region was the likely binding site for CKB, since binding was not affected when the SOCS box was removed. The identification of CKB as a possible target protein of Asb-9, the presence of Elongins B and C and Cullin-5, and the proposed function of the Asbs as ECS-type E3 ubiquitin ligases prompted further analysis into the biochemical consequences of the Asb-9-CKB interaction. Using transient and stable transfection techniques as well as immunofluorescence and confocal microscopy, we established that Asb-9 overexpression dramatically reduced endogenous CKB protein. Furthermore, the interaction resulted in the SOCS box-dependent ubiquitination and proteasomal degradation of CKB. These results suggest that Asb-9 may coordinate a novel molecular mechanism for the post-translational regulation of cellular CKB.

Asb-9 is one of 18 members of the ankyrin repeat-containing SOCS box protein family (Asbs). The amino acid sequence of murine Asb-9 predicts a 290-amino acid peptide, composed of a short N-terminal region of ~30 amino acids, followed by a series of six ankyrin repeats (amino acids 31–223) and a C-terminal SOCS box (amino acids 236–290). Although the Asb family represents the largest family of all SOCS box-containing...
proteins, their biological and biochemical functions remain poorly defined. Ankyrin repeats are a structural motif involved in protein-protein interactions (reviewed in Ref. 15), whereas the SOCS box interacts specifically with Elongin C. Several SOCS box-containing proteins act as part of an E3 ubiquitin ligase complex with the specificity of the complex determined by the protein interaction motif located upstream from the SOCS box (reviewed in Ref. 5).

Asb-9 appears to function in a similar manner to SH2-containing SOCS proteins, since the key players that are involved in the SOCS-mediated protein degradation pathway are also present in the Asb-9-CKB complex, specifically Cullin-5 and Elongins B and C. The SH2-containing SOCS proteins target key signaling proteins, such as the Janus kinases and receptors for degradation by the proteasome, thereby attenuating cytokine and tyrosine kinase receptor signaling. Our results demonstrate that, similarly to the other SOCS box-containing proteins, specific interaction between Asb-9 and CKB occurs independently of the SOCS box and that the interaction of Asb-9 with CKB leads to a SOCS box-dependent polyubiquitination of CKB and a decline in cellular CKB levels. Furthermore, recent studies suggest that Asb proteins regulate a number of biological processes by this mechanism (25, 26). For example, Asb-3 was reported to attenuate TNF-R2 signaling by directly targeting TNF-R2 for ubiquitination and proteasomal degradation. Cellular responses, such as TNF-R2-mediated Jun N-terminal kinase activation and apoptosis in response to TNF-α, were inhibited by Asb-3 (25).

In this paper, we report that Asb-9 is expressed in murine testes and kidneys with low expression in heart and liver. The expression pattern of CKB overlaps with that of Asb-9 in some tissues but is most highly expressed in tissues with high and fluctuating energy demands, such as the brain. We could not detect Asb-9 mRNA in murine brain in our studies, although expression has been reported in the hypothalamus (GenBank™ accession number BB173163.1). Interestingly, a detailed investigation examining creatine kinase isoforms in the brain revealed that CKB was expressed selectively in astrocytes among glial populations and was exclusive to inhibitory neurons among neuronal populations (45). CKB expression was very low in excitatory neurons. It was proposed that low CKB expression in excitatory neurons could be due to an increased turnover rate of CKB in these cells (45). It remains to be examined whether Asb-9 contributes to this highly regulated cellular distribution of creatine kinase enzymes. Antibodies generated in this study could be further optimized to allow the analysis of possible interactions between Asb-9 and CKB in brain as well as other tissues.

CKB is overexpressed in a wide range of solid tumors and tumor cell lines and has been used as a prognostic marker of cancer and metastasis, although this application remains controversial (27). The CKB gene is positively regulated by the oncogene E1a and negatively regulated by the tumor suppressor gene, p53 (29, 32). Also, many growth factors and hormones, such as estrogen, stimulate CKB activity and expression (46, 47). Estrogen has been shown to highly induce expression of creatine kinase B in the female rat reproductive tract as well as in human breast tumors and tissues (48). It is not known which factors induce the expression of Asb-9, but it is possible

**FIGURE 7.** Creatine kinase B is not detectable in 293T cells that overexpress Asb-9. 293T cells were either untransfected or transiently transfected with FLAG-Asb-9, FLAG-Asb-9ΔSB, FLAG-Asb-3, or FLAG-SOCS-3. Expression of the FLAG-tagged protein was analyzed by immunofluorescence using confocal microscopy with rat anti-FLAG/anti-rat-Cy5 antibody (red). Endogenous CKB was visualized with rabbit anti-CKB/anti-rabbit Alexa Fluor 488 antibody (green). 293T cells express high levels of CKB. However, upon overexpression of Asb-9, CKB could no longer be detected. Co-localization of CKB with all other expressed FLAG proteins was observed. DAPI, 4′,6-diamidino-2-phenylindole.
that regulators of CKB might also exert effects on Asb-9 activity and expression. It has been proposed that the CK system is involved in tumor growth through regulation of ATP production or modulation in an as yet undefined manner. Molecules that disrupt this system may have an impact on tumor growth or progression. Given the interaction of Asb-9 with CKB and implied roles of Asb-2 and Asb-8 in cancer, it is tempting to speculate that Asb-9 may also have a role in tumor development, but this will require further study.

It is clear that the physiological significance of the Asb-9-CKB interaction needs to be further examined. A thorough in vivo investigation utilizing genetically modified mouse models will extend the biochemical analyses presented in this paper. This work is currently in progress and will be essential in defining the biological setting of this novel interplay between Asb-9 and creatine kinase B.

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