Mechanisms of the Copper-dependent Turnover of the Copper Chaperone for Superoxide Dismutase*

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The copper chaperone for superoxide dismutase (CCS) is an intracellular metallochaperone required for incorporation of copper into the essential antioxidant enzyme copper/zinc superoxide dismutase (SOD1). Nutritional studies have revealed that the abundance of CCS is inversely proportional to the dietary and tissue copper content. To determine the mechanisms of copper-dependent regulation of CCS, copper incorporation into SOD1 and SOD1 enzymatic activity as well as CCS abundance and half-life were determined after metabolic labeling of CCS+−/− fibroblasts transfected with wild-type or mutant CCS. Wild-type CCS restored SOD1 activity in CCS−/− fibroblasts, and the abundance of this chaperone in these cells was inversely proportional to the copper content of the media, indicating that copper-dependent regulation of CCS is entirely post-translational. Although mutational studies demonstrated no role for CCS Domain I in this copper-dependent regulation, similar analysis of the CXC motif in Domain III revealed a critical role for these cysteine residues in mediating copper-dependent turnover of CCS. Further mutational studies revealed that this CXC-dependent copper-mediated turnover of CCS is independent of the mechanisms of delivery of copper to SOD1 including CCS-SOD1 interaction. Taken together these data demonstrate a mechanism determining the abundance of CCS that is competitive with the process of copper delivery to SOD1, revealing a unique post-translational component of intracellular copper homeostasis.

Copper is an essential nutrient functioning in facile electron transfer reactions critical for cellular respiration, iron oxidation, pigment formation, connective tissue biogenesis, peptide amidation, neurotransmitter biosynthesis, and antioxidant defense (1). This essential role of copper as well as the potential toxicity of this metal is revealed by the inherited disorders of copper metabolism, Menkes disease, and Wilson disease (2). These diseases result from loss-of-function mutations in genes encoding homologous copper-transporting P-type ATPases that reside in the secretory pathway mediating copper incorporation into nascent cuproproteins and copper efflux from the cell (3, 4). Copper-dependent trafficking of these ATPases provides a rapid, post-translational mechanism for the response of the cell to changes in copper content and is critical for intracellular copper homeostasis (5).

Under physiologic circumstances, the availability of intracellular copper is extraordinarily restricted due to the presence of excess chelating capacity (6). Thus, the delivery of copper to specific pathways within the cell is mediated by metallochaperones that function to provide copper directly to target proteins while protecting this metal from intracellular scavengers (7–9). At least three distinct pathways exist in mammalian cells that involve unique copper chaperones in this delivery process (10). Copper/zinc superoxide dismutase (SOD1) is a ubiquitous cytoplasmic enzyme that requires a single bound copper ion to catalyze the disproportionation of superoxide critical to the antioxidant defenses of all cells (11). The delivery and incorporation of copper into SOD1 in mammals and yeast is mediated by the copper chaperone for superoxide dismutase (CCS) via direct protein-protein interaction that involves a CCS-SOD1 heterodimeric intermediate (12–14).

Despite this detailed understanding of the mechanisms of copper trafficking and delivery within cells, virtually nothing is known about the homeostatic mechanisms that determine the hierarchy of copper acquisition by specific chaperones. After cellular uptake of copper by the plasma membrane transporter Ctr1, copper is presumably distributed to specific sites dependent upon intracellular requirements as determined by metabolic and enzymatic needs (15). Recent nutritional studies have demonstrated that the intracellular abundance of CCS is dependent upon the availability of dietary copper, suggesting a more direct link between copper availability and homeostasis (16, 17). In support of this concept, recent in vitro studies by L’Abbe and Bertinato (18) now directly demonstrate copper-dependent regulation of CCS abundance in rodent and human liver cell lines. In this study we have utilized the availability of cells derived from CCS−/− mouse embryos to directly examine the mechanisms of this process.

EXPERIMENTAL PROCEDURES

Materials—General chemicals and reagents were purchased through Sigma. DNA restriction and modifying enzymes were purchased from Promega and used according to the manufacturer’s specifications. Hybridization membranes and immunoblot blot reagents were purchased from Bio-Rad. Protein A beads were purchased from Repligen. Hybridization membranes and immunoblot blot reagents were purchased from Bio-Rad. Protein A beads were purchased from Repligen. Polyclonal rabbit antisera to human CCS were prepared and used for immunoblot analysis and immunoprecipitation as previously described (13). Polyclonal rabbit antisera to human actin was purchased from Sigma and murine monoclonal antisera to green fluorescent protein (GFP) was purchased from BD Biosciences. [35S]Methionine/[35S]cysteine Translabel and [35S]cysteine were purchased from MP Biomedicals Inc. 64Cu (700 Ci/mmol) was obtained by fast neutron bombardment of an enriched zinc target and used previously described (19).

Cloning and Mutagenesis—A full-length cDNA encoding wild-type human CCS (12) was cloned into the bicistronic mammalian expression vector pIRE-S2-EF-GFP (Clontech). The coding region corresponding to amino acids 86–274 was amplified by PCR and subcloned into pIRE-S2-EF-GFP as previously described (13). Site-directed nucleotide mutagenesis was performed in pIRE-S2-EF-GFP containing the entire human CCS.

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The abbreviations used are: SOD, superoxide dismutase; GFP, green fluorescent protein; MEF, mouse embryonic fibroblast; BCS, bathocuproine disulfonic acid; CCS, copper chaperone for superoxide dismutase.

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open reading frame using the QuikChange site-directed mutagenesis kit (Stratagene), 5′ sense and 3′ antisense oligonucleotides corresponding to the mutations C22S,C25S, C244S,C246S, and Y134E,G135E. In all cases, the presence of specific mutations and fidelity of the entire cDNA sequence were verified by automated fluorescent sequencing (PerkinElmer Life Sciences).

Cell Culture and Transfection—Immortalized Ctrl+/− and Ctrl−/− embryonic fibroblasts were obtained from E7 embryos and cultured as previously described (20). CCS−/− and SOD1−/− mice were obtained utilizing transgenic methodology as previously described (21, 22). CCS+/+ and CCS−/− mouse embryonic fibroblasts (MEFs) from E12.5 embryos were isolated and cultured in basal media. After the first passage MEFs were genotyped by PCR analysis as described. Freshly isolated MEFs from SOD+/+ and SOD−/− E12.5 embryos were isolated, allowed to adhere to tissue culture plates for 4 h, and then used immediately for metabolic experiments.

CCS−/− cells were routinely cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, and 7 × 105 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) and 2.5 μg of specified plasmid as outlined in the manufacturer’s specifications. In some experiments, after transfection cells were pooled and split into medium containing Dulbecco’s modified Eagle’s medium with 1% fetal bovine serum and either 200 μM bathocuproine disulfonic acid (BCS) or 150 μM CuCl2 for the subsequent 48 h. In all experiments the effectiveness of the treatment of cells with BCS and copper was confirmed by assaying SOD1 activity, previously shown to change with these agents, and by direct measurement of the intracellular copper content after treatment.

Metabolic Labeling and Immunoprecipitation—For copper labeling cells were incubated in Opti-MEM media containing 200 μCi/ml 64Cu for 2 h followed by collection of media and lysate as described previously (19, 23). Samples were then prepared in Laemmli buffer without β-mercaptoethanol and subjected to electrophoresis in an 8% SDS-polyacrylamide gel, and gels were exposed directly to a PhosphorImager screen (Molecular Dynamics). To determine the half-life of CCS in MEFs from SOD+/+ and SOD−/− mice, cells were initially incubated in cysteine methionine-deficient medium and subsequently pulsed for 1 h in this medium containing 50 μCi/ml [35S]cysteine (19). Samples were then subjected to 12.5% SDS-PAGE and exposed to a PhosphorImager screen. Signal intensity was determined using SigmaScan Pro 5.0 software (Systat). These experiments were replicated twice, and in all cases one embryo was used per cell culture for metabolic labeling, and at least three separate embryos from individual genotypes were used in each experiment to derive the half-life data. The half-life of wild type and mutant CCS in transfected CCS−/− MEFs was determined 60 h post-transfection with 48 h of treatment of 200 μM BCS or 150 μM CuCl2 as indicated in 1% fetal bovine serum media. Metabolic labeling with [35S]methionine/[35S]cysteine Translabel confirmed the absence of new CCS protein synthesis at this starting time point by immunoprecipitation analysis. In some experiments, 20 μl MG132 (Calbiochem) was added to the cell media at the beginning of the time period and maintained throughout all time points. Time points were then collected relative to the initial harvested sample, lysed, and subjected to immunoblot analysis as described below. Signal intensity was quantified using SigmaScan Pro 5.0 software and normalized to the 0-h time point. Data were graphed using Sigma Plot 8.0 (Systat).

Gel Filtration Chromatography—Gel filtration chromatography was performed at 4 °C with an AKTA fast protein liquid chromatography system and a Superose 12 column. (Amersham Biosciences) equilibrated with PBS. SOD1+/+ and SOD1−/− livers were isolated and homogenized as previously described (19). A total of 5 mg of liver lysates were applied to the column and eluted with phosphate-buffered saline in 0.5-ml fractions at a flow rate of 0.5 ml/min.

Immunoblot Analysis and SOD1 Activity Gels—Cell lysates were prepared in 50 mM HEPES, pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40, and 5 mM EDTA supplemented with Protease Inhibitor mixture (Calbiochem) on ice for 30 min followed by centrifugation for 10 min at 20,000 × g at 4 °C. Protein concentration for all samples was determined by the Bradford method (Bio-Rad). For specified experiments lysates were heated at 100 °C for 7 min and subjected to 12.5% SDS-PAGE. Gels were then transferred to nitrocellulose, and immunoblot analysis was carried out using the Super-Signal West Pico kit (Pierce) with goat anti rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibody (Pierce). Blots were then stripped in Restore buffer (Pierce) and analyzed in a similar fashion for actin at 1:5000 and GFP at 1:500. In all experiments for the determination of CCS abundance, differences in transfection efficiency and gel-loading were accounted for by quantification of GFP and actin expression, respectively, and then normalized accordingly. Fold difference was calculated by the normalized CCS signal intensities of BCS-treated samples divided by the copper-treated samples. One way analysis of variance statistical analysis using Dunnett’s method was used to determine statistical significance in -fold difference when compared with CCS+/+ cells. SOD activity gels were performed by electrophoresing lysates under non-denaturing, non-reducing conditions and then processing for SOD activity as previously described (19).

RESULTS

To examine the effect of intracellular copper content on CCS abundance, the steady state amount of this chaperone was determined by immunoblot analysis of embryonic fibroblasts derived from genetically altered mice. This analysis revealed a single 29-kDa band present in the lysate of CCS+/+ cells that is absent in CCS−/− lysates, confirming the specificity of the CCS antibody used in these studies (Fig. 1A, upper panel, lanes 1 and 2). The steady state abundance of CCS in Ctrl+/− cell lysates was markedly increased when compared with Ctrl−/− cells (Fig. 1A, upper panel, lanes 3 and 4). These differences in CCS signal intensity were not due to differences in protein concentration or gel loading as immunoblot analysis of actin showed equivalent intensities for all four cell lysates (Fig. 1A, lower panel). To confirm that the change in CCS abundance between Ctrl+/− and Ctrl−/− cells is due to the known differences in intracellular copper content between these cells (20), Ctrl+/− and Ctrl−/− cells were treated for 48 h with either 150 μM CuCl2 or 200 μM BCS, and then lysates were analyzed by immunoblot. In this circumstance chelation of copper with BCS increased the abundance of CCS in Ctrl+/− cells, eliminating the difference in CCS abundance seen previously between Ctrl+/− and Ctrl−/− cells (Fig. 1B, upper panel, lanes 2 versus 4). The addition of excess copper decreased the steady state level of CCS in both cell types (Fig. 1B, upper panel,
The abundance of wild-type CCS is similarly altered by intracellular copper dependence as observed in endogenously expressing CCS+/− cells. Restoration of copper incorporation into SOD1 in transfected CCS+/− cells was created and transfected into mouse embryonic fibroblasts. Although these studies provide strong evidence for a Ctr1-independent mechanism of copper-dependent CCS regulation, elucidation of the precise mechanisms of Ctr1 copper delivery to CCS may provide further insight into this process. These differences in CCS abundance were not due to differences in protein concentration or gel loading as determined by actin immunoblot analysis (Fig. 1B, lower panel).

The above findings demonstrate that genetic differences in cellular copper uptake are sufficient to alter intracellular CCS abundance and are consistent with recent observations examining the effect of copper on CCS abundance in liver cell lines (18). To dissect the mechanism of this process, SOD1 activity and copper incorporation into SOD1 was assessed in CCS+/− fibroblasts transiently transfected with CCS cDNA. As shown in Fig. 2A, transfection of wild-type CCS into CCS−/− cells restored SOD1 activity to that observed in endogenously expressing CCS+/+ cells. This restoration of SOD1 activity was the direct result of CCS function, as metabolic labeling of these cells with 64Cu revealed restoration of copper incorporation into SOD1 in transfected CCS−/− cells (Fig. 2A, lower panel, lanes 2 versus 3). As observed previously (21), in all cases this 64Cu-labeled band corresponded to SOD1 by subsequent immunoblot analysis (data not shown). Fig. 2A, lane 2 (upper panel) reveals a minimal amount of SOD1 activity in the CCS−/− lysate, consistent with what has been observed previously (19, 21). Although it remains possible that this residual activity may in part be due to the CCS-independent pathway documented recently by Culotta and coworkers (24), we speculate that this is more likely due to in vitro reconstitution of SOD1 activity, as there is a complete absence of any detectable incorporation of 64Cu into SOD1 in vivo (Fig. 2A, lane 2, lower panel). The metabolic labeling of cells for the detection of copper incorporation into SOD1 is a sensitive assay for CCS chaperone function (19, 21) and is, therefore, also used throughout this current study.

These studies demonstrate that transfected wild-type CCS functions to deliver copper and restore SOD1 activity in CCS−/− cells. To determine whether the transfected wild-type CCS is subject to the same copper-dependent regulation observed above in endogenously expressing cells, transfected CCS−/− cells were treated for 48 h post-transfection with either 150 μM CuCl2 or 200 μM BCS followed by immunoblot analysis. As can be seen in Fig. 2B, when transfected into CCS−/− cells, the abundance of wild-type CCS is similar to that observed in the copper-treated when compared with the copper-chelated samples (lanes 3 and 4). Because the plasmid used in these experiments is bicistronic and also expresses GFP, in all such experiments transfection efficiency was accounted for by quantification of GFP signal intensity (see “Experimental Procedures”) (Fig. 2B, middle panel). Differences in protein concentration and gel-loading were determined by immunoblotting for actin (Fig. 2B, bottom panel). The results shown are representative of 11 independent experiments, and in all cases the copper-treated cells had significantly less wild-type CCS at steady state when compared with BCS treatment (Table 1).

The above data indicate that wild-type CCS transfected into CCS−/− fibroblasts is subject to the identical copper-dependent regulation observed in endogenous cells, providing an experimental system to examine the role of CCS in this process. Because the transfected plasmid contains only the open-reading frame of CCS, these findings also support the concept that this copper-dependent regulation is mediated by a process of post-translational protein turnover (18). As shown in Fig. 3A, the structure of human CCS consists of three unique domains that serve specific functions in the process of copper delivery to SOD1. Domain I includes the copper binding motif, MTQCS, thought to be critical for copper acquisition under the physiologically copper-limited conditions of the cell (25). To examine the role of Domain I in the copper-dependent turnover of CCS, a Domain I deletion mutant Δ1–86 CCS was created and transfected into CCS−/− cells. Consistent with a role for this domain in CCS copper acquisition in mammalian cells, transfected cells were deficient in SOD1 activity when compared with cells transfected with wild-type CCS (Fig. 3B, upper panel, lanes 3 and 4). This loss of SOD1 activity was the result of impaired copper delivery by CCS, as CCS−/− cells transfected with Δ1–86 CCS lacked 64Cu-labeled SOD1 when compared with cells transfected with wild-type CCS or endogenously expressing CCS+/+ cells (Fig. 3B, middle panel, lanes 4 versus 1 and 3). The absence of SOD1 activity was not due to lack of Δ1–86 CCS expression as immunoblot analysis revealed that the abundance of this protein was equivalent to that observed in cells transfected with wild-type CCS (Fig. 3B, lower panel, lanes 3 and 4). As anticipated from previous studies in yeast (25), this loss of function of Δ1–86 CCS was due to impaired copper binding as a C22S,C25S CCS mutant as altering the cysteines in the MTCQSC motif also failed to restore SOD1 activity in transfected CCS−/− cells (data not shown).

In Saccharomyces cerevisiae, the role of CCS Domain I in copper acquisition can be bypassed with increased exogenous copper in the media (25). The experimental system shown here allowed this same concept to be tested in mammalian cells, and therefore, SOD1 activity was examined in CCS−/− cells transfected with wild-type or Δ1–86 CCS and treated for 48 h with either 150 μM CuCl2 or 200 μM BCS. Δ1–86 CCS and CCS−/− lysates showed no observable SOD1 activity in both untreated and BCS-treated samples (Fig. 3C, lanes 2, 3, 5, and 6). Although SOD1 activity was detected in the cells transfected with Δ1–86 CCS and treated with excess copper (Fig. 3C, lane 1), this SOD1

### TABLE 1

| Condition | αCCS activity (U/mg) | Replicates |
|-----------|----------------------|------------|
| WT CCS    | 3.5 ± 0.1            | 3          |
| Δ1–86     | 3.5 ± 0.1            | 3          |
| C244S,C246S | 1.0 ± 0.2         | 8          |
| Y134E,G135E | 2.2 ± 0.9         | 4          |

### FIGURE 2

Transfection of CCS cDNA in CCS−/− mouse embryonic fibroblasts. A, lysates from CCS+/+ cells, CCS−/− cells, and CCS−/− cells transiently transfected with a plasmid encoding wild-type human CCS (WT CCS) were analyzed for SOD1 activity (upper panel) and 64Cu incorporation into SOD1 (lower panel) as described under "Experimental Procedures." B, CCS−/− cells, CCS−/− cells, and CCS−/− cells transiently transfected with a plasmid encoding wild-type human CCS (WT CCS) were treated post-transfection for 48 h with either 150 μM CuCl2 or 200 μM BCS as described under "Experimental Procedures." Equivalent amounts of protein from each sample were analyzed by immunoblot with CCS (upper panel), GFP (middle panel), and actin (lower panel) antibodies.
activity is independent of CCS function as similar SOD1 activity was observed in the untransfected CCS−/− cells exposed to excess copper (Fig. 3C, lane 4). This observation is consistent with prior observations in CCS−/− mice and recent studies by Culotta that demonstrate a CCS-independent pathway for copper incorporation into SOD1 (24).

Although the data in Fig. 3C reveal that in contrast to yeast CCS, Domain I of human CCS is required for CCS-dependent copper incorporation into SOD1 in mammalian cells, Domain I plays no role in the copper-dependent regulation of CCS abundance as immunoblot analysis of Δ1–86 and wild-type CCS transfected into CCS−/− cells and treated for 48 h with either 150 μM CuCl2, or 200 μM BCS revealed identical responses to copper conditions (Fig. 3D). Consistent with this immunoblot, quantitation of the results of multiple such experiments revealed an average -fold difference with the Δ1–86 CCS similar to that observed with wild type (Table 1).

Domain III of CCS is required for the insertion of copper into SOD1, and this is mediated by a CXC motif that binds copper and participates in the disulfide isomerase activity essential for metallation (26–28). Because Domain III is also required for interaction with SOD1, the function of this domain in the copper-dependent regulation of CCS abundance was examined by generating a C244S,C246S CCS mutant that was then transfected into CCS−/− cells. Consistent with previous findings in yeast, CCS−/− cells expressing C244S,C246S CCS showed no detectable levels of SOD1 activity above that seen in CCS−/− cells (Fig. 4A, upper panel), and this was due to the lack of incorporation of copper into SOD1 (Fig. 4A, middle panel). As for the previous experiments, the abundance of mutant and wild-type CCS was comparable by immunoblot analysis (Fig. 4A, lower panel, lanes 3 and 4). In contrast to the findings with Δ1–86 CCS, when CCS−/− cells expressing C244S,C246S CCS were treated for 48 h with either 150 μM CuCl2, or 200 μM BCS, no apparent difference in the steady state level of CCS was observed (Fig. 4B, lanes 3 and 4). This result is representative of eight separate experiments (Table 1) and was corrected for differences in transfection efficiency and gel-loading by examining expression of GFP and actin, respectively, in these same lysates (see "Experimental Procedures").

These data reveal the interesting finding that the same CXC motif that binds copper for SOD1 metallation is also involved in the copper-dependent regulation of CCS turnover. Because the cysteine residues in this motif also participate in formation of an interchain disulfide bond with SOD1 during copper insertion (27, 28), experiments were next designed to determine whether the copper-dependent regulation of CCS turnover requires interaction with SOD1. To examine this question, the steady state level of CCS was analyzed in SOD1−/− mice, where CCS is still able to bind copper but there is no available SOD1 with which to interact. As shown in Fig. 5A, liver lysates from SOD1−/− mice have significantly reduced levels of CCS at steady state compared with SOD1+/+ mice and wild-type littermates (7–9), and therefore, to determine whether the difference in CCS turnover observed in SOD1−/− mice results from an alteration in dimeriza-
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FIGURE 5. Abundance and turnover of CCS in SOD1−/− mice. A, 50 µg of protein from liver lysates of CCS+/−, CCS−/−, SOD1−/−, and SOD1−/− mice were analyzed for CCS expression by immunoblot analysis. B, E12 mouse fibroblasts were metabolically labeled with [35S]methionine/[35S]cysteine Translabel, and the half-life of CCS was determined by pulse-chase analysis after immunoprecipitation with CCS antibody as described under “Experimental Procedures.” The averages of multiple embryos for one experiment are shown with S.D., and data are plotted on log scale. C, 5 mg of SOD1−/− (filled circles and solid line) and SOD1−/− mouse liver lysates (open circles and dashed line) were subjected to gel filtration chromatography on a Superose 12 column, and subsequent fractions were analyzed by immunoblot for CCS as described under “Experimental Procedures.” The averages of multiple experiments are plotted.

FIGURE 6. Role of SOD1 interaction in copper-dependent regulation of CCS abundance. A, lysates from CCS−/− cells, CCS+/− cells, CCS−/− cells transiently transfected with a plasmid encoding wild-type human CCS (WT CCS), and CCS−/− cells transiently transfected with a plasmid encoding human CCS with mutations in the Domain II tyrosine and lysine critical for SOD1 interaction (Y134E,G135E) were analyzed for SOD1 activity (upper panel), 64Cu incorporation into SOD1 (middle panel), and CCS expression (lower panel) as described under “Experimental Procedures.” B, CCS+/− cells transiently transfected with plasmids encoding either wild-type human CCS (WT) or human CCS with mutations in the Domain II tyrosine and glycine critical for SOD1 interaction (Y134E,G135E); CCS−/− cells were treated for 48 h post-transfection with either 150 µM CuCl2 or 200 µM BCS, and lysates were analyzed for CCS expression. C, CCS−/− cells transiently transfected with plasmids encoding either wild-type human CCS (WT) or human CCS with mutations in the cysteines critical for copper binding in Domain III and the Domain II tyrosine and glycine critical for SOD1 interaction (Y134E,G135E + C244S,C246S) were treated for 48 h post-transfection with either 150 µM CuCl2 or 200 µM BCS, and lysates were analyzed for CCS expression as described under “Experimental Procedures.”

The above data suggest that interaction with SOD1 may not be critical for the copper-dependent regulation of CCS turnover. However, manipulation of copper levels could not be conducted in the SOD1−/− cells, as these do not survive past 48 h in vitro (29). Therefore, to address this question by a second experimental approach, a Y134E,G135E CCS mutant was constructed that has previously been demonstrated to impair interaction with SOD1 without disrupting the copper binding capacity of Domain III (26). As anticipated, Y134E,G135E CCS was unable to restore SOD1 activity above that seen in CCS−/− cells (Fig. 6A, upper panel), and this was due to impaired copper incorporation into SOD1 as demonstrated by 64Cu-labeling (Fig. 6A, middle panel). Interestingly, examination of the steady state abundance of the Y134E,G135E CCS consistently revealed lower amounts than transfected wild-type CCS (Fig. 6A, lower panel, lanes 3 and 4). Although this small decrease in Y134E,G135E CCS abundance cannot account for the absence of SOD1 activity in the transfected CCS−/− cells, additional experiments revealed that the turnover of Y134E,G135E CCS was increased compared with wild-type CCS (data not shown). As can be seen in Fig. 6B, Y134E,G135E CCS migrates slower than wild-type CCS, a finding consistent with similar observations of SOD1 missense mutants (30) and likely due to changes in the biophysical properties of the protein despite the reducing and denaturing conditions. As seen in Fig. 6B, when these transfected cells were treated with either 150 µM CuCl2 or 200 µM BCS for 48 h, the abundance of Y134E,G135E CCS was found to change in a manner consistent with the copper-dependent regulation observed for wild-type CCS (Table 1), indicating that interaction with SOD1 is not critical for the copper-dependent turnover of CCS. As noted above, the steady state level of Y134E,G135E CCS was decreased compared with the wild-type CCS under each condition (Fig. 6B), suggesting that as was observed in the SOD1−/− mice, copper binding by CCS in the absence of SOD1 interaction increases the copper-dependent turnover of CCS. Taken together, these data indicate that the principal determinant of copper-dependent turnover of CCS is copper binding at the CXC motif in Domain III. Consistent with this concept, the incorporation of the C244S,C246S mutations into Y134E,G135E CCS abrogated the copper-dependent turnover (Fig. 6C, lanes 3 and 4).

Previous studies by L’Abbé and Bertinato (18) using liver cell lines suggested that the copper-dependent regulation of wild-type CCS abundance is due to differences in CCS turnover involving proteasomal degradation. Because the plasmids used in this current study encode only the open reading frame of CCS, the transfection experiments into CCS−/− cells provide strong support for a mechanism involving copper-
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FIGURE 7. Mechanisms of CCS turnover in conditions of increased copper. A, CCS−/− cells were transiently transfected with a plasmid encoding either wild-type human CCS (WT) or human CCS with mutations in the cysteines critical for copper binding in Domain III (C244S,C246S) and maintained in media with 200 μM BCS or 150 μM CuCl2, and the half-life of CCS was determined by immunoblot analysis at specific time points after transfection absent of new protein synthesis from the transfected plasmids as described under “Experimental Procedures.” B, in some experiments CCS−/− cells transiently transfected with a plasmid encoding wild-type human CCS and maintained in 150 μM CuCl2 were incubated with 20 μM MG132 (WT + MG132) during the time course of half-life analysis as described under “Experimental Procedures.” A representative immunoblot (B) is shown.

FIGURE 8. Model of copper-dependent regulation of CCS abundance. As determined in previous studies (26, 28, 29), apoCCS acquires copper from the intracellular milieu and delivers this metal to SOD1 by a series of intra- and intermolecular transitions involving copper movement from CCS Domain I to CCS Domain III and then SOD1. The data in this current study reveal that the identical Domain III cysteines in CCS required for copper binding and insertion into the target SOD1 also mediate the copper-dependent turnover of CCS. In this diagram this process is illustrated most simply as direct binding of copper to this motif followed by subsequent ubiquitin-mediated proteasomal degradation (Ub), consistent with the turnover data presented here and shown previously for endogenous CCS in cell lines (18). The model reveals the central role of this process in copper homeostasis.

The results of this study reveal that the process of copper-dependent turnover of CCS is kinetic and involves copper homeostasis. To directly examine this process, wild-type and C244S,C246S CCS were transfected into CCS−/− cells, and the half-life was determined as indicated under “Experimental Procedures.” Consistent with the studies noted above, the half-life of wild-type CCS in CCS−/− cells was significantly decreased under conditions of excess copper, and this increased turnover was abrogated by the addition of the proteasomal inhibitor MG132 (Fig. 7, A and B). In support of the concept that the principal determinant of copper-dependent turnover of CCS is the CXC motif in Domain III, half-life analysis of the C244S,C246S CCS mutant in CCS−/− cells revealed a complete absence of the effect of copper on CCS turnover (Fig. 7A).

DISCUSSION

The results of this study reveal that the process of copper-dependent regulation of CCS abundance is competitive with the pathway of copper delivery to SOD1, a finding that suggests a homeostatic mechanism regulating the balance between SOD1 activity and intracellular copper availability and distribution. This process would appear specific to CCS as the steady state level of the copper chaperone Atox1 is unaltered by similar changes in copper (data not shown). Although it remains to be determined how copper is distributed among the different chaperones once internalized via Ctr1, data have suggested that copper delivery to CCS is kinetically facile with very few steps, perhaps even a direct interaction between Ctr1 and the chaperone (19). Data gathered on CCS abundance in Ctrl−/− fibroblasts demonstrate that the copper-depend-ent turnover of CCS does not occur through a mechanism involving Ctr1 as the level of CCS was found to be regulated by copper in the Ctrl−/− fibroblasts (Fig. 1).

The data in this study demonstrate that copper-dependent regulation of the intracellular abundance of CCS requires the cysteines in the CXC motif of Domain III critical for copper insertion into SOD1 (Fig. 4). Although these cysteines are essential for both copper incorporation into SOD1 and copper-dependent turnover of CCS, these two processes are mechanistically distinct as demonstrated by deletion and mutational studies of CCS that abrogate copper delivery to, or interaction with SOD1 while maintaining copper-dependent regulation of CCS abundance (Figs. 3 and 6). The data further demonstrate that the mechanism of copper-dependent turnover of CCS does not involve alterations in the distribution of CCS monomer/dimer ratio in the cell as revealed by the gel filtration analysis in the SOD1−/− and SOD1−/− liver lysates (Fig. 5C). Indeed, both this analysis and the studies of the Y134E,G135E mutant CCS (Fig. 6) that is unable to homodimerize indicate that the process of copper-dependent turnover likely involves recognition of the CCS monomer. Furthermore, these studies indicate that the mechanism of copper-dependent turnover is unlikely to involve stable interaction with other cellular proteins as revealed by the identical elution profile of purified CCS and the CCS detected in the SOD1−/− and SOD1−/− liver lysates.

The reconstitution of SOD1 activity in CCS−/− fibroblasts transfected with wild-type CCS demonstrates that the transfected protein is
expressed in its proper conformation and is capable of acting as a copper chaperone. The copper-dependent regulation of transfected CCS independently confirms that the mechanism for this phenomenon is post-translational since expression from plasmid-based cDNA gave the same results as those observed in endogenously expressing cells (Fig. 2B) and cell lines (18). Although the qualitative changes in CCS abundance under the copper altered conditions used in this study were consistently reproduced, differences in the absolute amount of CCS were observed (see Table 1). Because the cell number and media requirements were kept identical in each experiment, this variation in the absolute amount of CCS under such conditions likely reflects reported differences in the amounts of available copper for chelation in unique subcellular locations that may change during physiological events such as the cell cycle (31). Consistent with this concept, statistical analysis of the -fold differences in Table 1 illustrates that C244S,C246S CCS was the only mutant where the change in CCS abundance with copper content was statistically significant when compared with the endogenous CCS+/+ cells. Intriguingly, in the experiments involving CCS−/− cells, the transfection efficiency averaged about 2.5%, whereas the restoration of SOD1 activity and Cu incorporation into SOD1 averaged about 59% that seen in CCS+/+ cells (see, for example Fig. 2A). This finding reflects an increase in the abundance of CCS per cell in transfected versus wild-type cells, and whereas it has no effect on our conclusions regarding the mutants, it does reveal that under these conditions CCS is rate-limiting for the formation of holoSOD1, an observation consistent with our previous finding that a substantial portion of SOD1 within the cytoplasm exists as an available pool of apoSOD1 (19).

The data also reveal a mechanistically interesting issue with CCS-dependent copper delivery to SOD1 in mammalian cells. Although Domain I is not required for copper delivery to SOD1 in yeast under conditions of excess copper (25), the situation is clearly different in mammalian cells. No SOD1 activity or copper incorporation into SOD1 was observed in CCS−/− cells transfected with the Δ1–86 CCS (Fig. 3B). Although SOD1 activity is restored under copper excess conditions (Fig. 3C), this must be due to a CCS-independent pathway of copper delivery to SOD1 (24) as both transfected and untransfected CCS+/+ cells have equivalent activity (Fig. 3C, lanes 1 and 4). These findings indicate that Domain I of CCS is required for CCS-dependent copper delivery to SOD1 in mammalian cells. Although studies have shown that deletion of Domain I of yeast CCS can still bind copper in vitro and interact with SOD1 by yeast two-hybrid (25), in vivo SOD1 incorporation studies (Fig. 3) clearly show that Domain I is required for active SOD1, and further structural and mechanistic studies will be needed to discern the precise role of Domain I in copper delivery to SOD1 in mammals.

Taken together our findings suggest a unique model of intracellular cytoplasmic copper homeostasis with specific relevance to SOD1 activity (Fig. 8). At steady state, the amount of copper bound to CCS may be minimal as the exchange of copper from CCS to SOD1 is very rapid (19). When the level of copper increases, a more substantial increase in the pool of holoCCS, defined here as CCS with copper bound at the CXC site, will occur, and will consist with the turnover studies in the presence of proteasome inhibition (Fig. 7) as well as the work of L’Abbe and Bertinato (18), the known conformational change upon copper binding to the CXC motif (25) may result in CCS ubiquitination and subsequent degradation. Such a hypothesis is consistent with the decrease in CCS observed in the absence of SOD1 (Fig. 5) or impaired interaction with SOD1 (Fig. 6), each of which would also increase the abundance of holoCCS. Intriguingly, although Domain I is required for CCS-dependent SOD1 activity, this same domain is not necessary for the copper-dependent turnover of CCS (Fig. 3D). This observation is also consistent with our proposed model, as under conditions of excess copper this metal will be bound to the CXC motif independent of Domain I transfer (25), once again increasing holoCCS and making this available for turnover. Future studies will now focus on the biochemical requirements for this copper-dependent turnover, including the role of the proteasome in the copper-dependent CCS turnover and the identification of the mechanisms of ubiquitination hypothesized above.

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