The Copper Chaperone for Superoxide Dismutase*

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Copper is distributed to distinct localizations in the cell through diverse pathways. We demonstrate here that the delivery of copper to copper/zinc superoxide dismutase (SOD1) is mediated through a soluble factor identified as Saccharomyces cerevisiae LYS7 and human CCS (copper chaperone for SOD). This factor is specific for SOD1 and does not deliver copper to proteins in the mitochondria, nucleus, or secretory pathway. Yeast cells containing a lys7Δ null mutation have normal levels of SOD1 protein, but fail to incorporate copper into SOD1, which is therefore devoid of superoxide scavenging activity. LYS7 and CCS specifically restore the biosynthetic and catalytic activity of SOD1 in vivo. Elucidation of the CCS copper delivery pathway may permit development of novel therapeutic approaches to human diseases that involve SOD1, including amyotrophic lateral sclerosis.

Superoxide dismutase I (SOD1)1 is a homodimeric zinc- and copper-containing enzyme that catalyzes the disproportionation of superoxide (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$) and O$_2$ in a reaction mediated through the cyclic reduction and oxidation of the bound copper ion (1). Recently, mutations in human SOD1 have been linked to the fatal motor neuron disorder, amyotrophic lateral sclerosis (ALS), more commonly known as Lou Gehrig’s disease (2, 3). Dominantly inherited mutations in SOD1 are responsible for this form of the disease (4–9), and evidence to date suggests that the toxicity from mutant SOD1 results from enhanced free radical-generating activity by the bound copper within the enzyme (10–12). Thus, degeneration in ALS may be prevented or ameliorated by interfering with copper incorporation into SOD1. However, testing of this hypothesis has been hampered by the lack of knowledge regarding the mechanism by which SOD1 acquires copper in vivo.

Recent studies indicate that the delivery of copper to specific proteins is mediated through distinct intracellular pathways of copper trafficking. We have previously identified a soluble copper carrier, yeast ATX1 and its human homologue HAH1, that specifically function in the delivery of copper to transport ATPases in the secretory pathway (13, 14). Tzagoloff and coworkers (15, 16) have identified a pathway involving a soluble yeast factor, COX17, that specifically deliver copper ions to cytochrome oxidase in the mitochondria. We therefore predicted that the insertion of copper into SOD1 would likewise involve a specific metal carrier or “copper chaperone.”

** Experimental Procedures

Cloning and RNA Analysis—Overlapping cDNA clones corresponding to ESTs homologous to LYS7 (GenBank™ accession numbers H58006 and AA085445) were obtained from Genome Systems (St. Louis, MO) and sequenced using a fluorescent dye terminator cycle sequencing kit (Perkin-Elmer) followed by analysis on a Perkin-Elmer ABI 373A XL sequencer. A 1068-base pair cDNA sequence was identified containing an 822-base pair open reading frame, which was termed CCS (GenBank™ accession number AF002110). RNA blot analysis was performed as described previously (13) using commercially obtained membranes containing poly(A)⁺ RNA from different human and fetal tissues (CLONTECH) or isolated from human cell lines.

To construct the CCS expressing plasmid pS5MC5, the coding region of human CCS was amplified by PCR and inserted at the EcoRI and BamHI sites of pSM703, placing CCS under the control of the Saccharomyces cerevisiae PKG1 (phosphoglycerokinase) promoter. The pHALT7 plasmid expressing the epitope-tagged LYS7-HA protein was obtained by first amplifying LYS7 sequences –440 to the stop codon by PCR using a downstream primer that converted the termination codon to a NotI site. This modified LYS7 fragment was then inserted at the ApoI and NdeI sites of the LEU2 CEN vector, pJ25AHα, resulting in the in-frame fusion of LYS7 to two copies of the HA epitope from influenza virus (17, 18). Construction of the sod2Δ::URA3 plasmid pGSO2D was as follows: S. cerevisiae SOD2 sequences –680 to –80 with respect to the start codon were amplified by PCR and directionally inserted at the BamHI and HindIII sites of pRS306, an URA3 integrating vector (19). SOD2 sequence +560 to +1700 were then directionally inserted in this plasmid at SpeI and BamHI sites. The resultant pGSO2D plasmid was linearized by digestion with BamHI and used to delete the chromosomal SOD2 gene of strain 1783 (20) by a one-step gene deletion (21), creating strain JS9002.

SOD Activity Gels—For analysis of SOD1 activity by NBT (nitro blue tetrazolium), cells were grown in 50 ml of YPD medium (22) to an A$_600$ of 1.0, were harvested and washed once in cold H2O, and the resultant cell pellet resuspended in 0.5 ml of a lysis buffer containing 10 mM sodium phosphate (pH 7.8), 1.0 mM EDTA, 0.1% Triton, 20 μg/ml leupeptin, 10 μg/ml pepstatin, and 1.0 mM phenylmethylsulfonyl fluoride. Cells were then broken by glass bead homogenization and glycerol in a reaction mediated through the cyclic reduction and oxidation of the bound copper ion (1). Recently, mutations in human SOD1 have been linked to the fatal motor neuron disorder, amyotrophic lateral sclerosis (ALS), more commonly known as Lou Gehrig’s disease (2, 3). Dominantly inherited mutations in SOD1 are responsible for this form of the disease (4–9), and evidence to date suggests that the toxicity from mutant SOD1 results from enhanced free radical-generating activity by the bound copper within the enzyme (10–12). Thus, degeneration in ALS may be prevented or ameliorated by interfering with copper incorporation into SOD1. However, testing of this hypothesis has been hampered by the lack of knowledge regarding the mechanism by which SOD1 acquires copper in vivo.

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were detected using as secondary antibody, anti-rabbit donkey IgG, at a 1:10,000 dilution (Amersham Corp.). LYS-HA was detected using a monoclonal anti-HA antibody from mouse (Bethyl Laboratories) diluted 1:1000 and as secondary antibody, sheep anti-mouse IgG (Amersham Corp.). All three secondary antibodies were conjugated to horseradish peroxidase and detection involved the HYBrite ECL kit (Amersham Corp.).

**RESULTS AND DISCUSSION**

A yeast genetic approach was utilized to identify the copper chaperone for SOD1. We predicted that a yeast mutant for the SOD1 copper chaperone would lack SOD1 activity and would exhibit the same phenotypes as a sod1Δ null mutant. Yeast cells lacking SOD1 are auxotrophic for both lysine and methionine when grown in air (26–28). We noted that the previously characterized lys7Δ mutant mimicked a sod1Δ mutation, and no SOD activity was associated with lys7Δ yeast. The expressed CCS gene failed to complement a sod1Δ mutation, and no SOD activity was associated with CCS (not shown). In comparison, the same CCS clone fully restored the aerobic growth of lys7Δ mutants on medium lacking lysine and methionine (Fig. 1), demonstrating that human CCS is a functional homologue to yeast LYS7.

To test whether LYS7 was essential for SOD1 activity, we examined SOD activity in lys7Δ cell lysates run on nonreducing polyacrylamide gels followed by autoradiography for 1 h to detect holoSOD1. Incorporation of 64Cu into FET3 was analyzed as described previously (13). Incubation with mouse anti-HA (Bethyl Laboratories) diluted 1:1000 proceeded for 2 h, and detection of LYS-HA was carried out with a FITC labeling kit (Boehringer Mannheim). All results are representative of three independent blots.

**FIG. 2. A**, sequence alignment of *S. cerevisiae* LYS7 and human CCS. The deduced amino acid sequence of CCS was aligned with LYS7; regions of amino acid identity are indicated by shading, and the putative copper binding motifs are shown in the boxed region. **B**, expression of the human CCS gene. RNA from the indicated tissues or cell lines was hybridized using a 32P-labeled CCS-specific cDNA probe and exposed to film for 24 h. This analysis revealed a major CCS-specific transcript of 1.5 kilobases as well as multiple higher molecular weight species in all tissues and cell lines as shown. Identical transcripts were observed in RNA samples from 31 additional human adult and fetal tissues, including multiple regions of the central nervous system. Results are representative of three independent blots.

**FIG. 1.** Oxygen-dependent lysine and methionine auxotrophy of lys7Δ mutants. The indicated strains of yeast were plated onto complete synthetic dextrose medium (22) lacking either (“LYS”) or methionine (“MET”) and were incubated for 3 days at 30 °C either in air (“+oxygen”) or in a BBL anaerobic culture jar (“−oxygen”). Strains utilized (shown in clockwise): wild type = 1783 (20); sod1Δ = KS105 (1783 sod1Δ::TRP1) (20); wild type = SY1699 (29); lys7Δ = SY2950 (SY1699 lys7Δ::LEU2) (29); lys7Δ-pLYS7 and lys7Δ-pCCS = SY2950 transformed with the pSL2502 plasmid harboring *S. cerevisiae* LYS7 (29) and with pSMCCS-expressing human CCS from the yeast PGK1 promoter, respectively.
FIG. 3. LYS7, but not ATX1, is required for copper insertion into SOD1. A and B, lysates from the indicated strains were subjected to electrophoresis on a 12% polyacrylamide gel under nonreducing, nonreducing conditions (A) or in the presence of SDS (B). A, assay for SOD activity was conducted using NBT. Positions of the mitochondrial manganese-containing SOD2 and the copper zinc SOD1 are indicated. B, Western blot analysis of extracts using an antibody directed against S. cerevisiae SOD1. Strains utilized: sod2Δ = JS002 (see A; sod1Δ = KS005 (20); wild type = SY1699 (29); lys7Δ = SY2950 (29). Results are representative of four independent lysates examined. C, autoradiograph of 64Cu-labeled SOD1 in cytosolic fractions from the indicated yeast strains following nondenaturing, nonreducing polyacrylamide gel electrophoresis. lys7Δ mutants are shown transformed with vector alone or plasmids containing either CCS or ATX1. D, biosynthesis of holoFET3 in crude membrane fractions from these same 64Cu-labeled strains. Results are representative of three independent experimental trials, and the apparent increase in FET3 labeling in cells expressing CCS is consistent.

In this analysis, wild type cells exhibited a single 64Cu-labeled protein corresponding to SOD1 (Fig. 3C), confirmed by Western blotting of this same gel (data not shown). Notably, lys7Δ mutants were devoid of 64Cu-labeled SOD1, and this defect was complemented by a episomal plasmid harboring human CCS (Fig. 3C). Thus, LYS7 is essential for copper incorporation into SOD1, and this function is conserved in the human homologue, CCS.

The subcellular localization of LYS7 was examined using a HA epitope-tagged protein. When expressed on a low copy centromeric vector, LYS7-HA fully complemented a lys7Δ mutation (not shown) and was observed to migrate as a single band on SDS gels (Fig. 4A). Extracts from cells expressing LYS7-HA were resolved into soluble and membrane-associated components, and as expected for a soluble protein, LYS-HA co-fractionated with cytosolic SOD1 to the supernatant, but was absent in the membrane fraction containing the proton ATPase (PMA1; Ref. 37) (Fig. 4A). When examined by immunofluorescence microscopy, LYS7-HA exhibited diffuse staining throughout the cytosol and was largely excluded from the nucleus. LYS7 is therefore cytosolic, as is the case for its recipient of copper delivery, SOD1.

LYS7 is highly specific for SOD1. The protein is not needed for copper trafficking to cytochrome oxidase in the mitochondria, as lys7Δ mutants are not defective for electron transport (data not shown). Additionally, LYS7 does not deliver copper to the nucleus for the regulation of metallothionein genes by the ACE1 trans-activator (38, 39); metallothionein gene transcription is unaffected in lys7Δ mutants (not shown). LYS7 also does not participate in the delivery of copper to the secretory pathway, as lys7Δ cells were found to be fully functional for incorporating 64Cu into FET3 (Fig. 3D). As additional evidence for the specificity of the LYS7 pathway, overexpression of another copper chaperone, ATX1, could not rescue the SOD1 copper labeling defect of lys7Δ mutants (Fig. 3C). Hence, distinct pathways of copper trafficking are mediated by ATX1 and LYS7. Although the molecular nature of the specificity of the ATX1/HAH1 and LYS7/CCS copper trafficking pathways has not yet been assessed, it is likely to involve protein-protein interactions between the metal chaperone and its copper-requiring recipient. For example, the homology of CCS with human SOD1 may facilitate metal transfer from the MTCXCC metal binding site in CCS to the copper binding site in SOD1.

Taken together, the studies presented here demonstrate that copper incorporation into SOD1 in intact cells is absolutely dependent on the presence of LYS7/CCS. Recent studies suggest that under normal conditions, approximately one-third of the total cellular SOD1 exists as the apoprotein (40). Thus copper loading of SOD1 by LYS7/CCS may represent a novel method of controlling enzyme activity at the posttranslational level. It is noteworthy that purified apoSOD1 will readily reconstitute with copper in vitro (1, 41, 42). Why is there a requirement for LYS7 in vivo? Presumably, the presence of multiple copper binding factors in an intact cell limits available copper, thus creating the need for a SOD1-specific chaperone, as well as chaperones for other copper proteins such as cytochrome oxidase in the mitochondria and the copper-transporting ATPases in the secretory pathway. It is therefore not surprising that copper chaperones such as ATX1, COX17, and...
LYS7 are found widespread among organisms as diverse as human and yeast (13, 43). Identification of the pathway of copper delivery to SOD1 may provide a useful approach to further examine the role of copper in SOD1-mediated familial ALS. For example, deletion of the murine CCS gene will permit direct testing of the role of aberrant SOD1 copper chemistry in motor neuron degeneration in transgenic mice expressing ALS mutant SOD1. As such, these studies may lead to the development of novel therapeutic approaches to prevent or ameliorate such injury in patients with SOD1-linked amyotrophic lateral sclerosis.

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