Copper Distributed by Atx1 Is Available to Copper Amine Oxidase 1 in Schizosaccharomyces pombe

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Copper amine oxidases (CAOs) have been proposed to be involved in the metabolism of xenobiotic and biogenic amines. The requirement for copper is absolute for their activity. In the fission yeast Schizosaccharomyces pombe, cao1* and cao2* genes are predicted to encode members of the CAO family. While both genes are expressed in wild-type cells, we determined that the expression of only cao1* but not cao2* results in the production of an active enzyme. Site-directed mutagenesis identified three histidine residues within the C-terminal region of Cao1 that are necessary for amine oxidase activity. By use of a cao1*GFP allele that retained wild-type function, Cao1-GFP was localized in the cytosol (GFP is green fluorescent protein). Under copper-limiting conditions, disruption of ctr4*, ctr5*, and cuf1* produced a defect in amine oxidase activity, indicating that a functionally active Cao1 requires Ctr4/5-mediated copper transport and the transcription factor Cuf1. Likewise, atx1 null cells exhibited substantially decreased levels of amine oxidase activity. In contrast, deletion of ccc2, cao1GFP, and pccs had no significant effect on Cao1 activity. Residual amine oxidase activity in cells lacking atx1* can be restored to normal levels by returning an atx1GFP allele, underscoring the critical importance of the presence of Atx1 in cells. Using two-hybrid analysis, we demonstrated that Cao1 physically interacts with Atx1 and that this association is comparable to that of Atx1 with the N-terminal region of Ccc2. Collectively, these results describe the first example of the assembly of Atx1 to act as a copper carrier for a molecule other than Ccc2 and its critical role in delivering copper to Cao1.

Copper is an essential metal ion cofactor that is required for many biological processes, including respiration, iron transport, superoxide anion detoxification, and assimilation of carbon and nitrogen sources (29). In excess, however, copper can participate in redox reactions that generate highly reactive oxygen species that cause damage to the cellular membrane, proteins, DNA, and RNA molecules (21). Consequently, organisms have evolved with multiple mechanisms to obtain sufficient levels of copper for incorporation into cuproproteins, while tightly controlling intracellular copper to avoid toxicity.

Copper amine oxidases (CAOs) have been identified in bacteria, yeasts, plants, and animals (39). Although little is known about their precise biological roles, CAOs from prokaryotes and lower eukaryotes can catalyze the oxidative deamination of several amine substrates to their corresponding aldehydes, providing carbon and nitrogen sources for cellular growth (7, 49). In higher eukaryotes, the situation is less well defined. Roles in wound healing, regulation of glucose uptake, metabolite signaling, and cell-cell adhesion and recognition, as well as detoxification of endogenous and xenobiotic amines, have been suggested for CAOs (42, 54). CAOs are dimeric proteins with molecular masses of ~140 to 190 kDa. CAOs have been shown to contain a single copper ion per monomer. Each copper ion is coordinated by the imidazole groups of three highly conserved histidine residues located in the C-terminal half of each monomer (43). In addition to the copper ion, each monomer contains a covalently bound cofactor, 2,4,5-trihydroxyphenylalanine quinone (TPQ), which is generated by posttranslational modification of the first conserved tyrosine residue (indicated in bold) in the consensus sequence Asn-Tyr-Glu (Asn/Glu)-Tyr (9, 12, 28, 36). The copper ion and oxygen are required for tyrosine modification into TPQ (10). An active CAO protein therefore has the capacity to convert a primary amine and molecular oxygen into the corresponding aldehyde, ammonia, and hydrogen peroxide. Currently, the pathways by which copper is delivered to CAOs are not well understood.

In the fission yeast Schizosaccharomyces pombe, the high-affinity copper uptake process occurs via a two-component copper transporting complex at the cell surface. This heteroprotein complex is composed of the Ctr4 and Ctr5 proteins, which are structurally related to each other and to the Ctr family of copper transporters (4, 46, 55). Ctr4 and Ctr5 are physically associated in vivo (55). Coexpression of both proteins is necessary for the proper function and localization of the heteroprotein complex at the plasma membrane (4, 55). Like most Ctr family members, the N-terminal hydrophilic domains of Ctr4 and Ctr5 are rich in methionine residues (32, 55). At the cell surface, these domains function independently to transport copper; however, both domains are required for optimal cell growth under copper-deficient conditions (4). The copper-sensing transcription factor Cuf1 plays an essential role in coordinating the copper-regulated transcription of copper transporter genes in S. pombe (2). Cuf1 activates transcription of the ctr4* and ctr5* genes under copper-deficient conditions and represses their expression under copper-replete conditions (5).

Once inside the cell, free-copper ions are virtually undetectable (47). One strategy by which cells transport copper to copper-dependent proteins within the cytoplasm involves the use of small soluble cytoplasmic copper carriers known as...
copper chaperones (19). In *Saccharomyces cerevisiae*, three small copper-binding proteins, Atrx1 (37),Css1 (16), and Cox17 (20), have been identified as chaperones to deliver copper to specific intracellular localizations. Atrx1 shuttles copper from the cytosol to post-Golgi vesicles by docking specifically with the Ccc2 copper-transporting P-type ATPase (45). Once transferred into the Golgi apparatus, it is thought that copper is loaded onto proteins as part of their maturation. In the mitochondrion, copper is stored mainly within the matrix, and from there copper is delivered by an as-yet-unidentified intracellular ligand in the mitochondrial intermembrane space. Once transported within the mitochondrial intermembrane space, copper is bound by Cox17, which in turn transfers copper into cytochrome c oxidase with the aid of Sco1 and Cox11 (13, 14, 24).

A third copper chaperone, Css1, donates copper specifically to the cytosolic copper zinc-superoxide dismutase (17). For the fission yeast *S. pombe*, although candidate molecules for copper trafficking into cells have been inferred from sequence homology to the *S. cerevisiae* chaperones (31), only one has been characterized experimentally, namely, Pccs (34). This protein is orthologous to the *S. cerevisiae*.Css1 cytosolic copper chaperone. In addition to its specific function to deliver copper to copper zinc-superoxide dismutase, Pccs is important for the survival of fission yeast cells in the continued presence of elevated concentrations of copper and cadmium (34). The latter observation indicates that some functional differences in copper chaperones may exist between the two species of yeast.

Examination of the *S. pombe* genome database suggests that the open reading frame (ORF)SPBC1709.10c encodes a putative ortholog of *S. cerevisiae* Atrx1. This putative ortholog bears 38% identity to Atrx1, and the copper-binding Met-X-Cys-X-X-Cys motif is conserved between the two proteins, suggesting a role for this protein in delivering copper within the cell. The Cox17 chaperone from *S. cerevisiae* has a related protein in *S. pombe* encoded by SPBC26H8.14c. This ortholog exhibits 38% identity to Atx1, and the copper-binding Met-X-Cys-X-X-Cys motif is conserved between the two proteins. Thus, it is likely that the Cox17-like protein from fission yeast might function in delivering copper to the mitochondrial cytochrome c oxidase.

In contrast to *S. pombe*, *S. cerevisiae* does not have an endogenous CAO (9, 33, 35). However, it has been shown that heterologous expression of a CAO from another organism in *S. cerevisiae* generates a functional enzyme (8, 33). We showed that, when an active CAO is expressed heterologously in *S. cerevisiae*, its production requires CTR-mediated copper transport and the transcription factor Mac1, which is essential for the expression of the high-affinity copper transport genes (33). Furthermore, we found that Atrx1 and, to a lesser extent, Ccc2 are required for the production of an active recombinant CAO in *S. cerevisiae* cells (33). While the results obtained with *S. cerevisiae* strongly suggest that the Atrx1 copper chaperone is important for delivering copper to cytosolic CAO, it remains to be established whether the *S. pombe* SPBC1709.10c gene, which encodes a putative ortholog of Atrx1, fulfills the same function in fission yeast. Given this point, we sought to examine the requirement of Atrx1 for CAO activity in *S. pombe*.

For fission yeast, two CAO molecules, SPA2EIP3.04 and SPBC1289.16c, have been annotated from the *S. pombe* Genome Project (52a). These proteins were initially designated SPAO1 and SPAO2 (33); however, recently they have been renamed Caao1 and Caao2, respectively, per the *S. pombe* Gene Naming Committee (http://www.genedb.org/gedeb/pombe /geneRegistry.jsp). In this study, we created *S. pombe* strains with single or double deletions of the *cao1* and *cao2* genes. We found that only the expression of *cao1* resulted in the production of an active enzyme capable of catalyzing the oxidative deamination of primary amines. *Cao2* played no apparent role in amine oxidase activity. Specific mutation of the His488, His460, and His227 residues to alanine within the C-terminal region of *Cao1* resulted in the complete loss of CAO activity. We determined that *Cao1* is a cytosolic protein which requires expression of genes involved in high-affinity copper transport to be active. Likewise, we determined that the presence of *S. pombe* Atrx1 conferred ~70 to 80% of the amine oxidase activity. By use of two-hybrid analysis, Atrx1 was shown to interact with Cao1. Collectively, these results demonstrate that the fission yeast Atrx1 protein can function as a copper chaperone for a molecule other than Ccc2, participating in the delivery of copper to Cao1.

MATERIALS AND METHODS

Yeast strains and growth media. The *S. pombe* strains used in this study were all isogenic derivatives of FY435 (h<sup>-</sup> his7-466 leu1-32 ura4-18ΔAdet-M210) (6) and included cuf1Δ (5), cao1Δ (FY435 plus cao1Δ::KAN<sup>r</sup>), cao2Δ (FY435 plus cao2Δ::KAN<sup>r</sup>), cao1Δ cao2Δ (FY435 plus cao1Δ::loxP cao2Δ::KAN<sup>r</sup>), cun4Δ ctn5Δ (4), atx1Δ (FY435 plus atx1Δ::KAN<sup>r</sup>), ccs2Δ (FY435 plus ccs2Δ::KAN<sup>r</sup>), cox17A (FY435 plus cox17Δ::KAN<sup>r</sup>), and pccsΔ (34) disruption strains. To ascertain that the results observed were not specific to the *S. pombe* strain FY435, identical experiments were carried out with the strain FY254 (h<sup>-</sup> cao1-1 leu1-32 ura4-18 Δadet-M210) and its isogenic derivatives cuf1Δ (32), cao1Δ (FY254 plus cao1Δ::KAN<sup>r</sup>), cao2Δ (FY254 plus cao2Δ::KAN<sup>r</sup>), ccs2Δ (FY254 plus ccs2Δ::KAN<sup>r</sup>), and cux17A (FY254 plus cux17Δ::KAN<sup>r</sup>). Double mutant gene disruptions were created by using a recyclable lox-P-KANMX2-loxP cassette through homologous recombination, as described previously (26). *S. pombe* cells were cultured in yeast extract plus supplements, which contains 3% glucose and 225 mg/liter of adenine, histidine, leucine, uracil, and lysine (1). To maintain plasmids in various strains, synthetic Edinburgh minimal medium (1) with 225 mg/liter of the required amino acids was used; unsupplemented Edinburgh minimal medium contains 160 nM copper. Conditions of copper deprivation or copper repletion were generated by adding the indicated concentration of ammonium tetrathiomolybdate (TTM) (323446; Sigma-Aldrich) or CuSO<sub>4</sub> to cells.

Plasmids. Plasmid pSP1*cao1* was constructed through a three-piece ligase by simultaneously introducing a 1,934-bp SpeI-SphI PCR-amplified fragment containing the *cao1* locus starting at position 1420 from the translational start codon up to position +514 after the initiator codon and a 2,060-bp SphI-APal PCR-amplified fragment bearing the *cao1*′ ORF starting at position +515 from the translational start codon up to position +436 after the stop codon into the SpeI-APal-digested pSP1 vector (15). To generate the *cao1*′ Stul-BspEI allele, a 12-bp Stul-BspEI linker was inserted in frame to and downstream of the last codon of the *cao1*′ gene by an overlap extension method (22). The insertion created four extra amino acid residues after the phenylalanine at position 712 (Ph712Arg-Pro-Ser-Gly-stop codon) of *Cao1*. This allele was found to be functional because of its ability to fully restore Cao activity in vivo. We used the restriction sites Stul and BspEI created within *cao1*′ to insert a copy of the GFP gene (30) (GFP is green fluorescent protein). The resulting plasmid was designated pSP1*cao1*′-GFP. To create the pBpadel6 *cao1*′-GFP plasmid, pSP1*cao1*′-GFP was digested with SpeI and included *cao1*′ (32), *cao2*Δ (FY254 plus *cao2Δ::KAN<sup>r</sup>), *cco2*Δ (FY254 plus *cco2Δ::loxP cco2Δ::KAN<sup>r</sup>), and *atx1*Δ (FY254 plus *atx1Δ::KAN<sup>r</sup>). Double mutant gene disruptions were created by using a recyclable lox-P-KANMX2-loxP cassette through homologous recombination, as described previously (26). *S. pombe* cells were cultured in yeast extract plus supplements, which contains 3% glucose and 225 mg/liter of adenine, histidine, leucine, uracil, and lysine (1). To maintain plasmids in various strains, synthetic Edinburgh minimal medium (1) with 225 mg/liter of the required amino acids was used; unsupplemented Edinburgh minimal medium contains 160 nM copper. Conditions of copper deprivation or copper repletion were generated by adding the indicated concentration of ammonium tetrathiomolybdate (TTM) (323446; Sigma-Aldrich) or CuSO<sub>4</sub> to cells grown to the mid-logarithmic exponential phase (A<sub>600nm</sub> of ~0.5). After treatment for 8 h, 20-ml samples were withdrawn from the cultures for subsequent detection of CAO activity or steady-state mRNA or protein analysis.
and Xhol and exchanged with a corresponding DNA region into the pSPCic0-1 plasmid. Likewise, for H621A and H627A mutants, the DNA sequence from each respective PCR-amplified fragment was digested with Xhol and BspEII and then used to replace the corresponding fragment from the plasmid pSPCic0-1.

All nucleotide changes that gave rise to mutations were verified by dideoxy sequencing. The atx1+ gene (SPBC707:10c) was isolated by PCR amplification using primers corresponding to the start and stop codons of the ORF from an S. pombe cDNA library (ATCC 87284; deposited by S. Elledge) (kind gift of Dennis J. Thiele, Duke University). Because the primers contained EcoRI and SalI restriction sites, the purified DNA fragment was digested with these restriction enzymes and cloned into the corresponding sites of pBluescript SK vector (Stratagene, La Jolla, CA). The resulting plasmid was named pSkat1-. Subsequently, the S. pombe atx1+ promoter up to position +1336 from the start codon of the atx1+ gene was isolated by PCR amplification and then inserted into pSkat1- at the NotI and PstI sites. This pSkat1+ derivative was designated pSkprom-atx1+. The NotI-SalI DNA fragment was isolated from pSkprom-atx1+ and then inserted into the NotI-SalI cut pSP1 plasmid, creating the pSPPlat+ plasmid. Analysis of gene expression. Total RNA was extracted by a hot phenol method as described previously (11). RNA samples were quantified spectrophotometrically, and 15 μg of RNA per sample was used for an RNase protection assay, which was carried out as described previously (41). Plasmid pSkat1+ was created by inserting a 172-bp NotI-EcoRI fragment from the cao1+ gene into the same sites of pBluescript SK (Stratagene, La Jolla, CA). The antisense RNA hybrizes to the region between positions +4 and +176 downstream from the initiator codon of cao1+. To generate pSkcano1+, a 191-bp fragment from the cao1+ gene (corresponding to the coding region between positions +2083 and +2274) was amplified and cloned into the BamHI-EcoRI sites of pBluescript SK. Plasmid pSkano1+ harbouring the cao1+ gene was then used to linearize a 151-bp BamHI-EcoRI fragment of the atx1+ gene into the same restriction sites of pBluescript SK. The antisense RNA pairs to the region between positions +334 and +485 downstream from the A of the start codon of atx1+. Except for pSkano1+, which was linearized with NotI, all the above-mentioned constructs were digested with BamHI for subsequent labeling with [32P]UTP and T7 RNA polymerase.

Cao1 and Cao2 localizing. A chemiluminescence in-gel assay for CAO activity was carried out as described previously (33). Quantitative assessments of CAO activity were conducted as described by Holt and Palcic (23), with the following modifications. Cell lysates were quantified using the Bradford assay (6a), and equal amounts of cellular protein were subjected to a colorimetric assay. A “physiological HEPES” 2× stock solution (100 mM HEPES, 10 mM KC1, 4 mM CaCl2, 2.6 mM MgCl2, 200 mM NaCl) was prepared and then adjusted to a pH of 7.4 with a solution of 1 N NaOH. Before each assay, 2 mM 4-aminooantipyrine (A-4382; Sigma), 4 mM vanillic acid (H-3601; Sigma-Aldrich), and 16 U/ml of horseradish peroxidase (P-6782; Sigma) were dissolved in 2× “physiological HEPES” buffer to generate a chromogenic solution. A stock solution of ethylenediaminetetraacetic acid (80 mM) was prepared in water and used as a substrate. Typically, reactions were performed by adding equal volumes of the chromogenic solution (40 μl), dyehyme (40 μl), and cell lysates (equal amounts). When needed, Milli-Q water was added to reach a final reaction volume of 160 μl. Reaction mixtures were incubated at 37°C for 4 to 6 h. The appearance of red dye was measured using a spectrophotometer at 498 nm. Before each 4-aminooantipyrine–vanillic acid–peroxidase–linked assay, the chromogenic solution was tested by adding a small volume of diluted hydrogen peroxide (1:1,000) to 100 μl of the chromogenic solution to confirm that the expected red dye was generated by the colorimetric assay.

Immunoblotting. For Western blotting experiments, protein extracts were resolved on 8% sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, proteins were electroblotted onto polyvinylidene difluoride Hybond-P membranes (Amersham Biosciences) for 1 h at 4°C. Membranes were blocked for 2 h at 4°C in 5% powdered skim milk (Difco) in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% bovine serum albumin) with 0.1% Tween 20 (TBST). After being washed in TBST, membranes were incubated with primary antibodies in 1% powdered skim milk in TBST at 16 h at 4°C. The following primary antisera were used for immunodetection: monoclonal anti-His, antibody (penta-His; Qiagen), monoclonal anti-GFP antibody (B-2; Santa Cruz Biotechnology), and monoclonal anti-PCNA antibody (PC10; Sigma). After incubation with the primary antibodies, membranes were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), developed with ECL reagents (Amersham Biosciences), and visualized by chemiluminescence. Microscopic analysis of Cao1 localization. Fluorescence microscopic analysis was performed as described previously (3). Fluorescence and differential interference contrast images of the cells were obtained with an Eclipse E800 epifluorescent microscope (Nikon, Melville, NY) equipped with an Orca ER digital cooled camera (Hamamatsu, Bridgewater, NJ). The samples were analyzed using ×1,000 magnification with the following filters: 465 to 495 nm (GFP) and 340 to 380 nm (DAPI [4’,6-diamino-2-phenylindole]). Cell fields shown in this study are representative of experiments repeated at least five times.

Two-hybrid interaction assay. S. cerevisiae strain L40 [MATa his3Δ200 trp1-901 leu2-3,112 ade2-LYS2 (Ura+) HIS3 Ura4Δ (leu2Δ his3Δ200 trp1-901)] (51) was used for two-hybrid analysis. For growth of cells, a modified synthetic minimal medium was used. This synthetic medium contains 83 mg/liter histidine, 83 mg/liter adenine, 30 mg/liter lysine, 2% dextrose, 50 mM MES [2-(N-morpholino)ethanesulfonic acid] buffer (pH 6.1), 10 μM MgSO4, 40 μM ZnCl2, 0.67% yeast nitrogen base minus copper and iron (MP Biomedicals, Solon, OH). To study the interaction of Caol with Atx1, the full-length version of the cao1+ ORF was inserted downstream of and in frame to the Escherichia coli lacZ gene as bait. PCR amplification of the cao1+ gene was carried out using primers corresponding to the start and stop codons of the ORF from S. pombe genomic DNA with Pfu DNA polymerase (Stratagene). To clone the PCR product into the pLexN-a vector (51), primers designed to generate EcoRI and SalI restriction sites at the upstream and downstream termini of the cao1+ gene were used. The PCR product was digested with EcoRI and SalI and cloned into the corresponding sites of pLexN-a. A similar cloning strategy was used to generate pLexN-a-CCA2-a, which contains the wild-type (WT) ccc2+ N-terminal codons 1 to 163. The prey plasmid pVP16-Atx1 was created by cloning a 207-bp BamHI-EcoRI DNA fragment containing the full-length coding region of atx1+ into the same sites of pVP16 (51). To create the atx1(RK) allele (see last paragraph of Results), primers corresponding to the beginning and the end of the atx1+ gene were made with mutations in the sequence that generated the Arg-20Glu, Arg-334Gly, and Arg-345Gly mutations, which were combined with the N-terminal and C-terminal regions of Atx1. Each L40 transformant strain harboring the indicated bait and prey plasmids was tested for the association of the two fusion proteins by a liquid β-galactosidase assay as described previously (56), except that cells were broken by vortexing in the presence of glass beads. The expression of the LexA-Cao1 and VP16-Atx1 fusion proteins were verified by immunoblot analysis using the following antisera: monoclonal anti-LexA antibody 2-12 (Santa Cruz Biotechnology) and monoclonal anti-VP16 antibody 1-21 (Santa Cruz Biotechnology). A monoclonal anti-phosphoglycerol kinase (PGK) antibody, 22C5-D8 (Molecular Probes), was used to detect PGK protein as an internal control.

RESULTS

Primary sequences of S. pombe CAOs. In S. pombe, there are two genes that encode proteins related to the CAO family of enzymes. We initially designated the SPAC2E1P1.04-04 and SPBC1289.16c-encoded proteins SPAO1 and SPAO2, respectively (33). However, because of potential name conflicts brought up by the S. pombe Gene Naming Committee, SPAC2E1P1.04 and SPBC1289.16c genes were renamed cao1+ and cao2+, respectively. The amino acid sequences of Cao1 and Cao2 are 57% and 40% identical, respectively, to that of the Hansenula polymorpha methylamine oxidase HPAO (Fig. 1). These represent the highest percentages of identity when Cao1 and Cao2 were compared to other prokaryotic and eukaryotic CAOs (C. Peter, J. Laliberté, and S. Labbé, unpublished data). Like HPAO and the other microbial and metazoan CAOs, both Cao1 and Cao2 have a tyrosine residue (Tyr409 for Cao1; Tyr394 for Cao2) that is contained within the highly conserved Asn-Tyr-Glu/Asp-Tyr sequence (Fig. 1). The first tyrosine within this sequence (indicated in bold) is known to be posttranslationally converted to TPQ by an autoxidative mechanism (9). The conversion of the precursor tyrosine to TPQ requires the presence of molecular oxygen and a mononuclear copper center. Based on the sequence alignment of Cao1 and Cao2 with HPAO, histidine residues (His456, His458, His460, His461, and His462) for Cao1; His465, His467, His469, and His464) for Cao2 may coordinate a single copper atom. Furthermore, the two amino acids (Tyr and Asp332) in Cao1
corresponding to HPAO Tyr305 and Asp319 are also conserved and might, by analogy with HPAO, serve to promote the active conformation of TPQ during the enzymatic reaction. In Cao2, however, a Phe residue is found at position 295 instead of Tyr. Despite this difference found in Cao2, amino acid alignments of Cao1 and Cao2 with the H. polymorpha protein HPAO and with CAOs from other organisms (data not shown) suggested that both Cao1 and Cao2 possess conserved motifs thought to be essential for CAO activity.

Expression of cao1/H11545 and cao2/H11545 is copper independent and is not regulated by Cuf1. As determined by RNase protection analyses, the steady-state mRNA levels of cao1/H11001 and cao2/H11001 in the WT strain FY435 were not regulated by either the copper chelator TTM (25 and 100 μM) or exogenous CuSO4 (10 and 100 μM) (Fig. 2). There were no significant changes in levels of cao1/H11001 and cao2/H11001 gene expression in treated cells compared to the basal levels in untreated cells (Fig. 2). As controls for signal specificity, cao1/H11001 and cao2/H11001 mRNAs were absent when RNA samples were isolated from cao1/H9004 and cao2/H9004 mutant cells, respectively (Fig. 2A and C). RNA levels in each experiment were quantitated (Fig. 2B and D). To further examine whether cao1+ and cao2+ transcription is controlled by the S. pombe regulatory transcription factor Cuf1, a cuf1/H9004 mutant strain was grown in the absence or presence of 25 and 100 μM TTM or

FIG. 1. Amino acid alignment of S. pombe Cao1 and Cao2 with H. polymorpha HPAO. Amino acid residues identical in the compared proteins are shown in inverse highlighting. The rectangle shown in the middle portion of the protein sequences indicates the location of the conserved N-Y-E-Y motif in which the first peptidyl tyrosine residue (black circle) is predicted to serve as a precursor for TPQ. Arrowheads indicate putative amino acids that may promote the active conformation of TPQ during the enzymatic reaction. Asterisks show putative histidine residues located in the C-terminal halves of the proteins that are potentially involved in the coordination of one copper atom. The amino acid sequence numbers refer to the position relative to the first amino acid of each protein.
10 and 100 μM CuSO₄. As shown in Fig. 2, the cuf1Δ mutant had no significant effect on the expression of the cao1⁺ and cao2⁺ genes. We therefore concluded that Cuf1, a transcription factor that is required for expression of genes involved in copper transport, is not required for constitutive transcription of the cao1⁺ and cao2⁺ genes.

**Cao1 is necessary for CAO activity in S. pombe.** Our previous studies of Cao1 and Cao2 took advantage of the fact that, although *S. cerevisiae* does not have an endogenous CAO, it can serve as an excellent host for the expression and characterization of genes encoding CAOs from other organisms (8, 33). We showed that, when heterologously expressed in *S. cerevisiae*, only cao1⁺ but not cao2⁺ resulted in the production of an active enzyme capable of catalyzing the oxidative deamination of ethylamine (33). To assess the amine oxidase activities of Cao1 and Cao2 in *S. pombe*, we used isogenic strains harboring WT cao1⁺ and cao2⁺ genes or insertionally inactive cao1Δ, cao2Δ, or cao1Δ cao2Δ double mutant genes. In the WT strain, we detected a strong chemiluminescent signal indicating the presence of an active amine oxidase (Fig. 3A). In contrast, no detectable CAO activity was observed in the cao1Δ mutant (Fig. 3A). The cao2Δ mutant behaved similarly to the WT strain and was equally competent in catalyzing the oxidative deamination of ethylamine. As expected,
no CAO activity was observed with the cao1Δ cao2Δ double mutant strain (Fig. 3A). To test whether transcripts of both cao1" and cao2" were present in WT or single or double deletion strains, the steady-state mRNA levels of cao1", cao2", and act1" were analyzed by RNase protection assays. The results shown in Fig. 3B indicate that, although Cao2 was inactive, its transcript was clearly detected in WT and cao1Δ strains. In contrast, no cao2" transcript was observed with the cao2Δ single mutant and cao1Δ cao2Δ double mutant strains (Fig. 3B). Further analysis showed that both the WT and the cao2Δ mutant strains expressed cao1", while the cao1Δ and cao1Δ cao2Δ mutants exhibited no cao1" transcripts (Fig. 3B). Taken together, our analysis of mutants defective in cao1", cao2", or both genes revealed that cao1" plays a unique role in producing amine oxidase activity in S. pombe.

In vivo mapping of critical histidine residues required for Cao1 function. Within the C-terminal half of Cao1, several His residues are conserved and may act as potential copper ligands. To ascertain their role in Cao1 activity, five His residues, His456, His458, His460, His462, and His627, were individually replaced with Ala (Fig. 4A). These mutations had no effect on the steady-state levels of Cao1 protein (Fig. 4B, middle). The WT and His mutant proteins were detected by immunoblotting using an anti-His5 antibody, due to the presence of an endogenous protein that contains 5 His residues located within the N-terminal residues 10 to 14 of Cao1 (Fig. 1). cao1Δ cells transformed with the vector alone exhibited no significant CAO activity (Fig. 4B and C). cao1Δ cells were transformed with WT cao1" or cao1" with mutations in various His residues and treated with 10 μM CuSO4, 20 μM TTM, and 100 μM TTM, Figure 4B and C show results obtained for cells treated with 10 μM CuSO4. Identical results were obtained when cells were treated with 20 μM TTM or 100 μM TTM (data not shown). In these experiments, expression of WT Cao1 restored CAO activity (Fig. 4B and C). In contrast, expression of the mutant alleles [cao1(H458A), cao1(H460A), and cao1(H627A)] in the cao1Δ cells failed to restore active CAO protein (Fig. 4B and C). On the other hand, we observed that cao1Δ cells expressing the cao1(H621A) allele displayed a CAO activity at a level similar to that of the WT cao1" allele expressed under the same conditions (Fig. 4B and C). Singularly, we noted that the Cao1 H456A mutant exhibited a much lower CAO activity, which is 5.9 times weaker than that of WT Cao1 (Fig. 4B and C). Taken together, these data reveal that the His456, His460, and His627 amino acid residues are necessary for Cao1 function, while replacement of the His456 amino acid residue with an Ala residue resulted only in a partially functional Cao1 protein.

Cellular localization of Cao1. To understand how Cao1 functions in S. pombe copper metabolism, we examined its cellular localization in living cells by fusing GFP to the C terminus of Cao1. A plasmid expressing the Cao1-GFP fusion protein was transformed into a cao1Δ mutant strain. Transformants were analyzed to determine the ability of the Cao1-GFP fusion protein to catalyze the oxidative deamination of ethylamine compared to the ability of the WT strain by use of the chemiluminescence activity assay. The results in Fig. 5A show that the Cao1-GFP fusion protein, expressed from its own promoter, retained CAO activity comparable to that of the WT, untagged Cao1 protein. By use of direct fluorescence microscopy, Cao1-GFP exhibited uniform fluorescent staining throughout the cytosol. Containing with DAPI indicated that Cao1-GFP was excluded from the nucleus (Fig. 5B, top). As a control, GFP alone produced from a recombinant construct showed a pattern of fluorescence throughout the cytoplasm and the nucleus (Fig. 5B, bottom). Thus, the absence of Cao1 from the nucleus strongly suggests that the protein is localized primarily in the cytoplasm when expressed in fission yeast.

Active Cao1 in S. pombe requires Ctr4/5-mediated copper transport and the transcription factor Cuf1. Cellular management of copper requires transport from the environment through the cellular membrane for delivery to copper-requiring enzymes. In S. pombe, ctr4+ and ctr5+ genes are known to encode two transmembrane proteins that form a tight complex localized at the cell surface (4, 55). Once assembled, this heteroprotein complex is required for high-affinity copper transport (55). The ctr4+ and ctr5+ genes are regulated by the copper-sensing transcription factor Cuf1 (2, 32). They are activated in response to copper deficiency and repressed under conditions of copper sufficiency. To ascertain the necessity of Cuf1 and the heteroprotein complex Ctr4/Ctr5 in supplying copper to Cao1, CAO activity was assayed with whole-cell extracts from WT, cuf1Δ, and ctr4Δ ctr5Δ cells. Under conditions of low copper supply, in the presence of 100 μM TTM, the growth medium, cuf1Δ or ctr4Δ ctr5Δ mutant cells exhibited no detectable CAO activity by use of an in-gel assay to detect enzymatic activity (Fig. 6A, left). In contrast, CAO activity was restored by supplementing the growth medium with 10 μM of exogenous copper (Fig. 6A, right). This is likely due to the accumulation of intracellular copper ions via a low-affinity copper uptake system, which bypasses the requirement for the S. pombe high-affinity Ctr4/Ctr5 copper transport system. To further confirm these results, we used a quantitative spectrophotometric method with 4-aminoantipyrine and vanillic acid (23) to determine whether deletions of cuf1+ and ctr4+ ctr5+ genes dramatically reduced CAO activity in the presence of 50 μM TTM (Fig. 6B, left). Consistent with the restoration of CAO activity by the addition of exogenous copper (10 μM CuSO4), we observed that copper-replete cuf1Δ and ctr4Δ cuf1Δ cells displayed an increase in CAO activity to 59 and 45% of the level observed for the WT strain, respectively (Fig. 6B, right). To ensure that the absence of CAO activity in the cuf1Δ and ctr4Δ cuf1Δ cells. Under conditions of copper deprivation was not due to a defect in Cao1 expression, we integrated a functional cao1"-GFP fusion allele into S. pombe cao1Δ, cuf1Δ, and cuf1Δ ctr4Δ ctr5Δ strains. As shown by two distinct CAO assays (Fig. 6C and D, left), the cao1Δ cuf1Δ and cuf1Δ ctr4Δ ctr5Δ mutant strains failed to display significant measurable CAO activity when cells were grown in the presence of the copper chelator TTM (50 or 100 μM). In contrast, when a cao1Δ disruption was transformed with the GFP epitope-tagged cao1" allele, high CAO activity was restored (Fig. 6C and D). Likewise, CAO activity was restored by the addition of 10 μM CuSO4 to the growth medium (Fig. 6C and D, right). To ascertain that the Cao1-GFP fusion protein was expressed in the cuf1Δ, cuf1Δ cuf1Δ, and cuf1Δ ctr4Δ ctr5Δ cells, total protein extracts from transformed cells were analyzed by immunoblotting (Fig. 6C, middle). These results demonstrated that Cao1-GFP was produced in the cuf1Δ cuf1Δ and cuf1Δ ctr4Δ ctr5Δ strains,
FIG. 4. Functional dissection of critical His residues in Cao1. (A) Schematic representation of the WT Cao1, Cao1 H456A, Cao1 H458A, Cao1 H460A, Cao1 H621A, and Cao1 H627A mutant proteins. The point mutations are marked with asterisks and an A (instead of the WT H residues). The black region indicates the location of the highly conserved consensus sequence NYEY, in which the first peptidyl tyrosine (Y) residue (black circle) serves as a putative precursor for TPQ formation. The active conformation of TPQ is presumably stabilized through interactions with Y307 and D321 in Cao1. The amino acid sequence numbers refer to the position relative to the first amino acid of the protein. (B) CAO activity was determined for cao1Δ cells that were transformed with a plasmid alone (−), WT cao1Δ, or the indicated mutant alleles of cao1 (top). Protein extracts were prepared from aliquots of cultures and then analyzed by immunoblotting using either anti-His5 or anti-PCNA (as an internal control) antibody. NS, nonspecific signal. (C) Total extracts from cells transformed as described for panel B were assayed for CAO activity using a spectrophotometric method with 4-aminoantipyrine and vanillic acid (23). The CAO activities reported represent the means from three separate determinations ± standard deviations.
indicating that the absence of CAO activity in these mutant strains was not due to the lack of Cao1-GFP expression. Collectively, these data indicate that under low-copper conditions, the production of active Cao1 in *S. pombe* requires the plasma membrane-associated high-affinity copper transporters Ctr4 and Ctr5, as well as the transcription factor Cuf1, which is necessary for the expression of the high-affinity copper uptake genes.

*S. pombe* Atx1-like protein is required for the synthesis of fully active Cao1. Copper is an essential trace element that is also toxic due to its proclivity to engage in redox reactions that generate detrimental reactive oxygen species (21). Consequently, organisms have evolved with cellular components that function to acquire and distribute copper in a carefully controlled fashion. The discovery of copper chaperones that are involved in intracellular distribution of copper is a typical example of this fine control (19). For *S. cerevisiae*, three distinct copper chaperones, Atx1 (37), Ccs1 (16), and Cox17 (20), have been identified and shown to deliver copper to distinct pathways or organelles. Based on the *S. pombe* genome database and published data, it has been hypothesized or proven that the copper chaperones, Atx1 (37), Ccs1 (16), and Cox17 (20), have been identified and shown to deliver copper to distinct pathways or organelles. Based on the *S. pombe* genome database and published data, it has been hypothesized or proven that the copper chaperones, Atx1, Ccs1, and Cox17, respectively. Furthermore, *S. pombe* atx1Δ (SPBC1709.10cΔ) mutant cells were defective for iron acquisition (data not shown), presumably because of a lack of copper incorporation into the ferroxidase Fio1 (31), which would prevent Fio1-Fip1 high-affinity iron transport activity at the plasma membrane. This result strongly suggests that the product of the fission yeast gene SPBC1709.10cΔ is a functional homolog of *S. cerevisiae* Atx1. To determine if one of the copper chaperones Atx1, Ccs1, and Cox17 was involved in delivering copper to Cao1, we generated mutants lacking each of the copper chaperone genes, as well as a mutant lacking a fourth gene, ccc2 (SPBC29A3.01), which is predicted to transport copper across intracellular membranes into the secretory pathway. The WT strain and the single mutants were tested for CAO activity. Strains were grown under both copper-limiting (50 or 100 μM TTM) and copper-replete (10 μM CuSO4) conditions. As shown in Fig. 7A and B (left), in the presence of TTM, atx1Δ cells exhibited a CAO activity much lower than those of WT cells and the other mutant cells. Using a spectrophotometric CAO assay, we determined that cells bearing an atx1 deletion displayed an activity ~2.6 to 3.2 times weaker than those of the WT cells or cells that harbored a ccc2, cott17, or pcs deletion (Fig. 7B). As expected, the decrease in CAO activity observed with the atx1Δ mutant cells was largely restored by the addition of 10 μM CuSO4 to the growth medium (Fig. 7A and B, right). To ensure that the diminution of CAO activity in an atx1Δ mutant was not due to the lack of expression of the Cao1 protein, we generated cao1Δ atx1Δ, cao1Δ ccs2Δ, cao1Δ cott17Δ, and cao1Δ pcsΔ double mutants. Subsequently, we integrated a functional cao1Δ-GFP allele expressed under the control of the cao1Δ promoter into the double disruption strains, as well as the cao1Δ single disruptant. The transformed strains were first grown in low-copper medium (50 or 100 μM TTM). Peroxidase-catalyzed chemiluminescence assays showed that the cao1Δ atx1Δ double mutant strain expressing cao1Δ-GFP appeared to have much less CAO activity than the other double mutants, even though the protein levels were comparable (Fig. 7C, left). The reduced level of CAO activity observed for cao1Δ atx1Δ cells was reversed by the addition of exogenous CuSO4 (10 μM) to the medium (Fig. 7C, right). These results were recapitulated by a second method that used a peroxidase-linked spectrophotometric CAO assay. As shown in Fig. 7D (left), cao1Δ atx1Δ double mutant cells expressing cao1Δ-GFP displayed ~70 to 80% less CAO activity under copper-limiting conditions. As expected, the mutant phenotype was largely corrected by the addition of copper to the growth medium (Fig. 7D, right).

Because the loss of function of the Atx1-like protein through gene inactivation resulted in less CAO activity, we reasoned that introduction of the WT atx1Δ locus on an episomal plasmid should restore CAO activity to WT levels. A CDNA fragment encompassing the atx1Δ gene was cloned into the pSP1 vector and transformed into two different atx1Δ mutant strains. While atx1Δ mutant strains carrying empty pSP1 exhibited low CAO activity under conditions of copper deprivation, the same strains expressing atx1Δ from the pSP1 plasmid displayed elevated levels of CAO activity that were comparable to those found in cells expressing an endogenous atx1Δ gene (Fig. 8). Taken together, these results reveal that optimal activity of the Cao1 protein requires a functional atx1Δ-like gene in fission yeast.
Caol1 associates with the Atx1 chaperone. Given the results that Caol1 requires the presence of the Atx1 protein to be fully active, we tested the possibility that Caol1 physically interacts with Atx1. We carried out two-hybrid experiments using the entire 712-amino-acid coding region of Caol1 fused in frame to that of the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as bait, whereas the coding region for the VP16 activation domain was fused to the DNA-binding domain of LexA as ba...
The five arginine and lysine residues with acidic glutamates resulted in an Atx1 molecule that was severely inhibited in its ability to interact with Cao1 [the Atx1(R,K) mutant] (Fig. 10). As a control, we monitored the physical interaction between the VP16-Atx1(R,K) mutant and LexA–Ccc2-a and found an ~84% decrease in the activity of the reporter gene product compared with those of the unaltered VP16-Atx1 and LexA–Ccc2-a fusion proteins (Fig. 10). Using Western blot analysis, we observed equivalent expression levels of the LexA-Cao1, LexA–Ccc2-a, VP16-Atx1, and VP16-Atx1(R,K) mutant fusion proteins regardless of the presence of copper or a copper chelator in the growth media (data not shown). Taken together, these results strongly suggest that Atx1 can act as a copper carrier for a molecule other than Ccc2, participating in the delivery of copper to Cao1.

**DISCUSSION**

Global analysis of the fission yeast genome indicates the presence of two candidate copper-containing amine oxidase genes, *cao1* and *cao2*, in *S. pombe*. Consistent with a role for Cao1 in the metabolism of primary amines as alternate sources of nitrogen to support growth, we have shown previously that the inability of *S. cerevisiae* to utilize ethylamine as the sole nitrogen source can be corrected by the heterologous expression of Cao1 from *S. pombe* in *S. cerevisiae* (33). Furthermore, we have observed that *S. pombe* cells harboring an inactivated *cao1* gene (*cao1*Δ) failed to grow in medium containing ethylamine as the sole source of nitrogen (Peter, Laliberte, and Labbé, unpublished). On the contrary, *cao1*Δ cells in which a WT *cao1* allele was reintegrated regained the capacity to utilize ethylamine as a nitrogen source (Peter, Laliberte, and Labbé, unpublished). On the contrary, *cao1*Δ cells in which a WT *cao1* allele was reintegrated regained the capacity to utilize ethylamine as a nitrogen source (Peter, Laliberte, and Labbé, unpublished). On the contrary, *cao1*Δ cells in which a WT *cao1* allele was reintegrated regained the capacity to utilize ethylamine as a nitrogen source (Peter, Laliberte, and Labbé, unpublished). On the contrary, *cao1*Δ cells in which a WT *cao1* allele was reintegrated regained the capacity to utilize ethylamine as a nitrogen source (Peter, Laliberte, and Labbé, unpublished). On the contrary, *cao1*Δ cells in which a WT *cao1* allele was reintegrated regained the capacity to utilize ethylamine as a nitrogen source (Peter, Laliberte, and Labbé, unpublished). On the contrary, *cao1*Δ cells in which a WT *cao1* allele was reintegrated regained the capacity to utilize ethylamine as a nitrogen source (Peter, Laliberte, and Labbé, unpublished).
tions, Cao1 may enable S. pombe cells to utilize primary amines as sources of nitrogen. The fact that S. cerevisiae does not express an endogenous protein homologous to Cao1 or to any of the members of the CAO family may reflect an important degree of divergence between the two yeasts with respect to environmental challenges they may have to cope with in their respective habitats. Alternatively, it may simply reflect a distinction between the biochemical pathways that are employed by these two yeasts to utilize carbon and nitrogen sources.

Although we detected CAO activity only in S. pombe cells expressing Cao1, we determined that both cao1/H11001 and cao2/H11001 genes were constitutively expressed under both copper-limiting and copper-replete conditions. Furthermore, we found that their expression was independent of cufl/H11001, a gene encoding the nutritional copper sensing trans inducer of the copper transport genes ctr4/H11001, ctr5/H11001, and ctr6/H11001 in fission yeast (5). Interestingly, cao1/H11001 and cao2/H11001 mRNA steady-state levels were reportedly induced by hydrogen peroxide (11). Because one of the by-products of CAO catalysis is hydrogen peroxide, this observation may suggest a mechanism for the positive transcriptional autoregulation of these genes, thereby ensuring their continuous expression.

The role of Cao2 in S. pombe is unclear because of the lack of CAO activity and the phenotype of the cao2Δ mutant. The lack of activity may be due to the substitution of Tyr for Phe$^{295}$ in the Cao2 protein. Based on the X-ray crystal structures of HPAO (36) and E. coli CAO (43), this conserved peptidyl Tyr residue is critical for the proper orientation of TPQ for optimal catalysis. Furthermore, we noticed that Cao2 contains a Cys$^{54} \rightarrow$ Ser amino acid modification. This amino acid substitution would prevent the formation of an important disulfide bridge that is highly conserved in all known eukaryotic CAOs (36), thereby potentially interfering with the correct topology of Cao2. Thus, these amino acid changes might render the Cao2 enzyme incapable of catalyzing ethylamine oxidation. On the other hand, perhaps we have not identified the appropriate amine substrate for Cao2. In this study, the primary amine substrates that we tested included monoamines (e.g., ethylamine), aromatic monoamines (e.g., benzylamine), and diamines (e.g., putrescine and 1,8-diaminooctane). None of these substrates were oxidized by Cao2 (Peter, Laliberte´, and Labbé, unpublished). In contrast, Cao1 catalyzed the oxidative deamination of all of the primary amines that we have tested. The best substrates for Cao1 were ethylamine, putrescine, and 1,8-diaminooctane, while the aromatic monoamine benzylamine was oxidized less efficiently (Peter, Laliberté, and Labbé, unpublished). Collectively, our data reveal that CAO activity was found in S. pombe cells expressing cao1/H11001 but not in cells expressing only cao2/H11001. It is possible, however, that the expression of cao2/H11001 may make a contribution under conditions not tested in our study. Further molecular and biochemical studies are needed to better define the role of Cao2.

Given the requirement of copper for TPQ formation in active CAOs, we sought to identify the cellular proteins involved in copper delivery to Cao1. The analysis of fission yeast cells in which both ctr4/H11001 and ctr5/H11001 genes have been inactivated demonstrated the loss of CAO activity under copper-limiting conditions. Likewise, the lack of Cuf1, the transcription factor that directs expression of the ctr4/H11001 and ctr5/H11001 genes, also results in the loss of CAO activity. As expected, the requirement of
Ctr4/Ctr5 or Cuf1 for CAO activity was bypassed by the addition of exogenous copper at concentrations equivalent to or exceeding 10 μM to the growth medium. The lack of CAO activity in these mutant strains underscores the requirement of copper delivered by the high-affinity copper uptake machinery for the synthesis of an active CAO protein.

To further explore the pathway by which copper is distributed to Cao1, we examined the effects of deleting the ccc2, ccc1, cox17, and pccs genes, which are predicted (or proven, for the pccs gene [34]) to be involved in delivering intracellular copper, on Cao1 activity. Under conditions of copper starvation, cells lacking Atx1 exhibited a substantial decrease (~70 to 80%) in CAO activity. In contrast, under the same conditions, the deletions of the other genes failed to show any significant inhibition of CAO activity. We also noticed that while Atx1 can be bypassed even under low-copper conditions (with ~20 to 30% CAO activity), a defect in atx1 affects Cao1 activation under both low-copper (100 μM TTM) and high-copper (10 μM CuSO4) conditions. We believe that the latter observation (independent of the presence of TTM) further supports a specific function for Atx1 in the activation of Cao1. A previous study of global analysis of protein localization in the fission yeast S. pombe revealed that Atx1 is localized throughout the cytoplasm and the nucleus (40). Because both Cao1 and Atx1 can colocalize in the cytoplasm, we tested the ability of Cao1 to physically associate with Atx1. Using yeast two-hybrid analysis, we detected a weak but highly reproducible interaction between Cao1 and Atx1. How this might occur? X-ray crystal structures of CAOs, including HPAO from H. polymorpha (the closest ortholog of Cao1), indicate that they form homodimeric molecules, with an overall structure reminiscent of a mushroom cap (18, 36, 38, 43). The bulk of the homodimer has two identical active sites arranged along a molecular twofold symmetrical axis. Each active site is located in the interior of the protein and harbors one copper ion and one TPQ cofactor, both at a very close distance to each other. The active site pocket forms one open channel. Interestingly, the cavity of the channel leading to the active site contains residues that promote favorable binding for positively charged molecules, such as compounds containing amine groups.

S. pombe Atx1 encodes a very small polypeptide of only 7.6 kDa. We observed that the corresponding Lys 24, Lys 28, Lys61, Lys 62, and Lys 65 residues, which generate a positively charged surface on the S. cerevisiae Atx1 protein, are either replaced by Arg (instead of Lys) for the first 2 residues or perfectly conserved for the last 3 residues in S. pombe Atx1. It is therefore possible that Atx1 docks on the cavity of the entry channel at the active site of Cao1, which is predicted to have the capacity to retain relatively large substrates, particularly those with multiple positively charged Lys residues with NH2 groups on their surface (27). In fact, it is known that peptides with exposed lysines can associate with and inhibit human Cao1/Vap1-dependent lymphocyte rolling activity (53). To support this hypothesis, using two-hybrid analysis (Fig. 10), we observed that changing the overall charge of the putative Atx1

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**FIG. 10.** The interaction between Cao1 and Atx1 is similar to that of Ccc2-a and Atx1 and is enhanced under low-copper conditions. (A) Schematic diagrams of the LexA DNA binding domain and fusions with Cao1 and the putative N-terminal cytosolic domain of the S. pombe Ccc2 polypeptide (Ccc2-a) are depicted at left. The VP16 and chimeric VP16-Atx1 and VP16-Atxx(R,K) molecules used as prey are shown at right. (B) Mid-logarithmic-phase cells cotransformed with the indicated plasmids were grown under basal or copper-deficient (1 mM TTM) conditions or with excess copper (100 μM CuSO4) for 5 h. Protein-protein interactions were detected by liquid β-galactosidase assays, and results are indicated in Miller units. Error bars indicate the standard deviations of samples analyzed in triplicate.
basic region from positive to negative, as well as removing the predicted exposed NH₂ groups from arginine and lysine residues, resulted in an Atxl molecule that showed a significant decrease (~70%) in physical association with Cao1. In addition to the Atxl lys-rich face, which is known to be important for the interaction between the chaperone and its target, the N terminus of Atxl harbors a Met-X-Cys-X₂-Cys copper-binding motif that is present in two copies at the N terminus of S. cerevisiae Ccc2. The current model proposes that the transfer of copper from Atxl to Ccc2 involves a direct metal ion exchange between the conserved Met-X-Cys-X₂-Cys motifs found in both proteins (25). Interestingly, S. pombe Cao1 has a Met-X-His-X₂-Cys sequence (amino acids 151 to 156), which is predicted to be located at the cell surface near the entrance to the active site of the protein. This prediction was made based on the HPAO crystal structure and visualized using the 3D Molecule Viewer program (Invitrogen Corporation, San Jose, CA). A similar amino acid sequence is also present in HPAO (amino acids 148 to 153), except that the His residue is found immediately after the Met residue. In contrast, no such sequence exists in Cao1. Further characterization of Cao1 is required to ascertain whether the protein lacking the Met-X-His-X₂-Cys sequence is unable to interact with Atxl and/or is less active under conditions of copper deprivation. It is also possible that Atxl delivers copper with the aid of an accessory protein, which would participate in the insertion of copper into Cao1; however, no such factor has yet been identified.

In S. pombe, the Atxl metallochaperone represents an important source of copper for Cao1. However, Atxl is not the only means by which Cao1 can be activated. As shown in this study, Cao1 can obtain copper in an Atxl-independent manner. The activity of Cao1 in yeast cells lacking Atxl was detectable and highly reproducible and ranged from ~20 to 30% of the Atxl-dependent activity. We used a classical genetic approach to identify additional trans-acting proteins responsible for Atxl-independent activation of Cao1. Different copper-binding proteins were examined, including the putative copper chaperones encoded by pccs and coxl7 and the copper-transporting P-type ATPase Ccc2. However, deletions of these genes had no significant effect on Cao1 activity. Furthermore, the requirement for glutathione, which is known to play an important role in copper homeostasis, was also studied. We found that mutant cells defective in glutathione biogenesis showed no marked decrease in Cao1 activity (Peter, Laliberté, and Labbé, unpublished). Thus, the identity of the other molecule(s) that may be involved in delivering copper to Cao1 in fission yeast remains to be established. Because related proteins exist in pathogenic fungi, the mechanisms of copper loading on Cao1 homologs are indeed an interesting area for future study.

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