The Schizosaccharomyces pombe Pccs Protein Functions in Both Copper Trafficking and Metal Detoxification Pathways*§

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Because copper is both an essential cofactor and a toxic metal, different strategies have evolved to appropriately regulate its homeostasis as a function of changing environmental copper levels. In this report, we describe a metallochaperone-like protein from Schizosaccharomyces pombe that maintains the delicate balance between essentiality and toxicity. This protein, designated Pccs, has four distinct domains. SOD activity assays reveal that the first three domains of Pccs are necessary and sufficient to deliver copper to its target, copper-zinc superoxide dismutase (SOD1). Pccs domain IV, which is absent in Saccharomyces cerevisiae CCS1, contains seventeen cysteine residues, eight pairs of which are in a potential metal coordination arrangement, Cys-Cys. We show that S. cerevisiae ace1Δ mutant cells expressing the full-length Pccs molecule are resistant to copper toxicity. Furthermore, we demonstrate that the Pccs domain IV enhances copper resistance of the ace1Δ cells by an order of magnitude compared with that observed in the same strain expressing a pccs+I-II-III allele encoding Pccs domains I-III. We consistently found that S. pombe cells disrupted in the pccs+ gene exhibit an increased sensitivity to copper and cadmium. Furthermore, we demonstrate that overexpression of pccs+ is associated with increased copper resistance in fission yeast cells. Taken together, our findings suggest that Pccs activates apo-SOD1 under copper-limiting conditions through the use of its first three domains and protects cells against metal ion toxicity via its fourth domain.

Copper is both an essential and yet toxic cellular constituent (1). As a redox metal, it is critical enzymes as a catalytic cofactor (2). However, this same property can render copper cytotoxic because of its ability to participate in Fenton-like reactions that can generate hydroxyl radical, which may in turn cause cellular damage (3). In order to provide organisms with sufficient copper while at the same time preventing copper toxicity, specialized mechanisms have evolved for its uptake, intracellular trafficking, and sequestration within cells (4, 5).

In recent years, the use of yeast as a model organism has revealed a wealth of new information on how cells establish and maintain copper concentrations that are compatible with their needs (6–8). Under copper-limiting conditions and following the reduction of Cu2+ to Cu+ by the Fre plasma membrane reductases (9–13), the budding yeast Saccharomyces cerevisiae transports copper ions into yeast cells using two separate high affinity permeases encoded by the CTR1 and CTR3 genes (14–18). Copper-limiting conditions increase levels of mRNA synthesized from the FRE1, CTR1, and CTR3 genes, whereas their expression is repressed under copper-replete conditions, thereby preventing copper accumulation during acute copper exposure (12, 19). The copper-responsive regulation of FRE1, CTR1, and CTR3 gene expression is mediated by the copper-sensing transcription factor Mac1 (12, 19, 20). In addition to transcriptional regulation, copper uptake is further regulated through the copper-dependent degradation of the Ctr1 transporter. Ctr1 is stable under conditions of copper deprivation, but is rapidly degraded in the presence of excess copper (21). Unlike Ctr1, however, the Ctr3 transporter is not affected by increased exogenous copper concentrations (17).

Consistent with the notion that there is little intracellular free copper available in the cytoplasm of the yeast cell (22), specialized metallochaperones have been identified, including Atx1 (23, 24), Cox17 (25–28), and CCS1 (29), that bind copper after it enters the cell. These chaperones subsequently distribute their copper cargo to specific intracellular proteins or compartments (30, 31). Atx1 is a 73 amino acid cytosolic protein with a predicted molecular mass of 8.2 kDa (23). This copper chaperone is known to coordinate a single metal ion via its Met-X-Cys-X-Cys motif (24). Atx1 shuttles copper from the cytosol to post-Golgi vesicles by specifically docking with the Ccc2 copper-transporting P-type ATPase (32). Once loaded, Ccc2 subsequently pumps copper into the lumen of the Golgi, to metallate the copper-dependent ferroxidase Fet3 (32, 33). Although ATX1 and CCC2 gene transcription is unaffected by intracellular copper status, these genes are transcriptionally

1 The abbreviations used are: CTR1, copper transporter 1; BCS, bathocuproinedisulfonic acid; CCS, copper chaperone for SOD1; Cuf1, copper factor 1; CuSE, copper-signaling element; MT, metallothionein; Pccs, S. pombe copper chaperone for SOD1; PCNA, proliferating cell nuclear antigen; SOD1, copper-zinc superoxide dismutase; YES, yeast extract plus supplements; GFP, green fluorescent protein.

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activated in response to iron starvation, illustrating their importance to iron metabolism through the delivery of copper to Fet3 (23, 34). Cox17, a 8.05-kDa copper chaperone, delivers copper specifically to the mitochondria for the assembly of cytochrome c oxidase (25, 35). Cox17 binds three Cu⁺ ions through cysteine residues that are arranged in a Cys-Cys-X-Cys configuration (27). Copper-loaded Cox17 has been shown to shuttle in and out of the mitochondrial intermembrane space and is thought to dock to at least one inner mitochondrial membrane protein, Sco1, for subsequent copper donation to the Cu₄ site of the cytochrome c oxidase (36–38). A third copper chaperone, CCS1, specifically activates SOD1 (29). CCS1 has a vacuolar copper transporter (56). In response to copper limitation, CCS proteins, CCS1 and CCS2, are up-regulated by copper-sensitive promoters (56, 57). Binding studies reveal that the CuF1 N-terminal 174 amino acids are important for binding to the CuSE (58). A motif containing five clustered cysteine residues near its C terminus constitutes the minimal copper-sensing module of CuF1 and serves to inactivate CuF1 function when cells are grown under copper-deplete conditions (59).

Upon uptake into fission yeast cells, copper ions are presumably taken up by putative copper chaperones that are as yet uncharacterized at the molecular level. Examination of the S. pombe Genome data base suggests that the open reading frame SPAC22E12.04 encodes a putative ortholog of the S. cerevisiae CCS1. Although this putative ortholog bears 30% identity and 47% similarity to its baker’s yeast counterpart, notable differences exist between the two molecules. For instance, the N-terminal domain I of the S. pombe CCS ortholog (designated Pcs) lacks the copper-binding Met-X-Cys-X-Cys motif. In addition, Pcs harbors an extra domain at the C terminus that contains a series of cysteine residues, which are arranged in Cys-Cys configurations. Given these differences between the S. pombe and S. cerevisiae CCS proteins, we sought to dissect the functional features of Pcs. When the pccs¹-IV and pccs¹-III alleles were ectopically expressed in a S. cerevisiae ccs1Δ strain, we found that cells producing a polypeptide spanning domains I-III displayed nearly wild-type levels of SOD1 activity. Under low basal copper conditions, S. pombe strains harboring a deletion of the pccs¹⁺ gene were defective in SOD1 activity. Transforming this strain with a plasmid expressing the first 222 amino acids of Pcs (domains I-III) restored SOD1 activity to the same level obtained with the full-length Pcs protein. When the full-length S. pombe pccs¹⁺ gene or a cDNA fragment that encodes only the Pcs domain IV was expressed in a S. cerevisiae ccs1Δ strain, these cells exhibited a copper-resistant growth phenotype in the absence of exogenous copper. Consistently, pccsΔ mutant cells were sensitive to copper and cadmium. Furthermore, overexpression of pccs¹-IV or pccs¹-IV alone conferred tolerance to elevated copper levels in fission yeast cells. Taken together, these results reveal that the S. pombe Pcs protein function in dual pathways to deliver copper to SOD1 during conditions of copper scarcity and to detoxify metal ions during conditions of metal excess.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—Six isogenic S. pombe strains, the wild-type FY254 (h⁻ can1-1 leu1-32 ade6-M210 ura4-D18) (59), cuf1α (h⁻ can1-1 leu1-32 ade6-M210 ura4-D18 cuf1Δ::ura4⁺) (54), pccsΔ (h⁻ can1-1 ade6-M210 ura4-D18 pccsΔ::ura4⁺), sod1Δ (h⁻ can1-1 ade6-M210 ura4-D18 sod1Δ::ura4⁺), zyn1Δ pccsΔ (h⁻ can1-1 leu1-32 ade6-M210 ura4-D18 zyn1Δ::ura4⁺), and zyn1Δ pccsΔ (h⁻ can1-1 leu1-32 ade6-M210 ura4-D18 zyn1Δ::ura4⁺ pccsΔ::KAN) were used in this study. To ascertain that the results seen were not specific to the S. pombe FY254 strain, identical experiments were conducted with the FY435 strain (h⁻ his7-366 leu1-32 ade6-M210 ura4-D18) (60). The pccsΔ (h⁻ his7-366 ade6-M210 ura4-D18 pccsΔ::KAN) and pccsΔ sod1Δ cuf1Δ (h⁻ his7-366 ade6-M210 ura4-D18 sod1Δ::ura4⁺ cuf1Δ::KAN) mutant strains are isogenic to the FY435 strain. S. pombe cells were grown in yeast extract plus supplements (YES) or in Edinburgh minimal medium with the necessary auxotrophic requirements (61). When pccsΔ and sod1Δ mutants were grown, Edinburgh minimal medium was further supplemented with 225 μg/l of adenine, histidine, leucine, methionine, lysine, and cysteine, unless otherwise stated. Under anaerobic growth conditions, fission yeast cells were grown in culture jars with BD GasPak EZ (BD Diagnostic System, Sparks, MD). For expression of the human CCS and S. pombe pccs⁺ genes in S. cerevisiae, the ccs1Δ (also named lys7Δ) mutant strain, denoted EGY103lys7Δ (MATα ura2-3, 11 his3-1 Δtrpl-
289 ura3-52 lys7-L1272 was utilized to ensure that within the cell, only the ectopically expressed hCCS or Pccs was the sole protein with the ability to deliver copper to SOD1. The EGY103lys7A strain was derived from the parent strain EGY103 (MATa leu2-3, 11 his3-1 Δtherp1-289 ura3-52) (62) by gene deletion and replacement. S. cerevisiae strain DTY59 (MATa his6 leu2-3, -112 ura3-52 ace1-225 cup1R-3) (63) was used to test the ability of Pccs domain IV to detoxify copper in bakers' yeast. DTY59 was derived from strain DTY7 (MATa his6 leu2-3, -112 ura3-52 CUP1R-3) as described previously (64). S. cerevisiae cells were grown in rich medium (1% yeast extract, 2% bactopeptone, 2% dextrose) or synthetic complete medium lacking histidine or uracil for strains transformed with plasmids harboring the HIS3 or URA3 gene, respectively.

Plasmids—The pccs' gene was isolated by PCR using primers corresponding to the start and stop codons of the open reading frame from an S. pombe cDNA library (ATCC 67284, deposited by S. Elledge) (generous gift of Dennis J. Thiele, Duke University, Durham, NC). To clone the PCR product into the pBluescript vector (65), the EcoRI and BamHI sites found in pccs' were modified by PCR mutagenesis, eliminating both sites without altering the amino acid sequence of the Pccs protein. The modified pccs' allele was re-amplified by PCR using primers designed to generate EcoRI and XbaI sites at the upstream and downstream termini of the open reading frame. The PCR product obtained was digested with EcoRI and XbaI and cloned into the corresponding sites of the centromeric yeast plasmid pRS313 to generate pRSspcs'I-II-III-IV. Subsequently, the S. cerevisiae CCS1 promoter up to 395 from the start codon of the CCS1 gene was subcloned into pRSspcs'I-II-III-IV at the XhoI and EcoRI sites. Similarly, a 335-bp XbaI-BamHI DNA fragment containing the 3' untranslated region of CCS1 was inserted into the same sites of pRSspcs'I-II-III-IV. The 666-bp EcoRI-XbaI fragment encoding the first three domains of Pccs was used to replace the EcoRI-XbaI fragment from plasmid pRSspcs'I-II-III-IV to produce the plasmid pRSspcs'I-II-III-IV. The human and S. cerevisiae CCS genes were obtained by PCR amplification using primers that contained EcoRI and XbaI restriction sites using a human HeLa cell cDNA library or genomic DNA from S. cerevisiae strain DTY7 as templates, respectively. The purified DNA fragments were digested with EcoRI and XbaI and subsequently cloned into the corresponding sites in plasmid pRSspcs'I-II-III-IV to replace the pccs'I-II-III-IV gene. To generate the pSP1sod1' plasmid, a 1508-bp BamHI-NotI PCR-amplified DNA segment containing the S. pombe sod1' locus starting at −860 from the translational start codon up to +273 after the stop codon was inserted into the BamHI and NotI sites of pSP1 (66). The pccs' cDNA was isolated using the S. pombe cDNA library described above. The purified DNA fragment that contained flanking BglII and Smal restriction sites was digested and cloned into the pBluescript SK vector (Stratagene, La Jolla, CA) at compatible BamHI and Smal sites. To create a plasmid that has the pccs' promoter driving the expression of the pccs' gene, the S. pombe pccs' regulatory region (positions −1105 to −1) was amplified by PCR and inserted just before the ATG codon of the pccs' gene using the NotI and SpeI sites. Subsequently, a Smal-PstI DNA fragment of the pccs' terminator up to +542 from the stop codon was isolated by PCR from the S. pombe FY254 genomic DNA. Once generated and verified by DNA sequencing, the DNA fragment containing the pccs' gene and its regulatory regions was isolated from the pSKspecs'I-II-III-IV plasmid using NotI and PstI and inserted into the corresponding sites of pSP1. The resulting plasmid was designated pSP1specs'I-II-III-IV. To generate the pccs'I-II-III-IV-StuI-BspEI allele, a 12-bp StuI-BspEI linker was inserted in-frame and downstream of the last codon of the pccs' gene by the overlap extension method (67). The insertion created four extra amino acid residues after the alanine at position 297 (Ala297→Arg-Pro-Ser-Gly-Stop) of Pccs. This allele was found to be functional because of its ability to fully restore SOD1 activity in vitro. We used the restriction sites StuI and BspEI created within pccs' to insert a copy of the gfp gene (68) or four copies of the Haemophilus influenzae hemagglutinin epitope (69). The plasmid, denoted pSP1specs'I-II-III-IV-GFP, was used to determine the localization of Pccs-GFP fusion protein in S. pombe by fluorescence microscopy.

![Sequence alignment of S. pombe Pccs with S. cerevisiae and human CCS](image)

**FIG. 1.** Sequence alignment of S. pombe Pccs with S. cerevisiae and human CCS. Amino acid residues that are identical in at least two of the compared proteins are indicated above the alignment. The domains are numbered I-IV. Domains I, II, and III are boxed in blue, green, and brown, respectively. The fourth domain of Pccs is underlined. The MXCXXC sequence in domain I and the CXC motif in domain III are depicted in red and indicated with a double line above the sequence. The dots indicate residues that are predicted to be involved in CCS-SOD1 protein-protein interactions (40).
similar strategy was utilized to generate the pccs-I-II-III-Stul-BspEI allele, except that the Stul-BspEI linker was placed in-frame at the end of a DNA fragment that encodes only the Pccs domains I, II, and III. After amplifying the plasmid pSP1pccs-I-II-III-GFP or pSP1pccs-I-II-III-zyml plasmid, respectively.

To ascertain if the expression of different versions of the pccs-I-II-III-zym1 alleles contributed to the increased copper resistance of S. cerevisiae DT59 cells, the p4XXGPD expression vectors were used as described previously (70). Using appropriate primers that contained Spel and SmaI sites, the pccs-I-II-III-IV, pccs-I-II-III, pccs-I-II, and pccs-I-I-III-zym1 alleles were isolated by PCR from the plasmids pSP1pccs-I-II-III-IV, pSP1pccs-I-II-III, pSP1pccs-I-II, and pSP1pccs-I-I-III-zyml, respectively. The PCR products obtained were digested with Spel and SmaI and cloned into the corresponding sites of p426GPD. To assess if overexpression of pccs-I-II-III-zym1 alleles can rescue the copper hypersensitivity of a ctr6Δ strain overexpressing the wild-type ctr6+ gene, plasmids pREP3X-pccs-I-II-III, pREP3X-pccs-I-II-III, pREP3X-pccs-I-II, and pREP3X-pccs-I-I-III-zyml were constructed as follows. Five DNA fragments encompassing the pccs-I-I-III-IV, pccs-I-I-III, pccs-I-II, and pccs-I-I-III-zyml alleles were PCR amplified with flanking XhoI and SmaI sites from the pSP1pccs-I-II-III-zyml plasmid. The resulting PCR products were digested with XhoI and SmaI and cloned into the corresponding sites of pREP3X (71, 72). For ectopic expression of the ctr6+ gene, the thiamine-repressible promoter system was used as described previously (56). Protein and Enzyme Assays—For Western blotting experiments, S. pombe and S. cerevisiae cells were grown to OD$_{600}$ of 1.0 in selective medium. Protein extracts were prepared from cells that were untreated or incubated for 3 h (S. cerevisiae) or 11 h (S. pombe) with either CuSO$_4$ (100 $\mu$M) or BCS (100 $\mu$M), and then quantitated as described previously (73). The extracts were resolved by SDS-polyacrylamide electrophoresis, transferred to polyvinylidene difluoride Hybond-P (Amersham Biosciences), and the immunoblots analyzed for steady-state levels of SOD by chemiluminescence. SOD1 activity assays were performed using in-gel nitro blue tetrazolium staining as previously described (54). Spectrophotometric determination of SOD activity was also performed using the protein extracts by measuring the inhibition of the reduction rate of cytochrome c by SOD, which competes for reactive oxygen species produced from the xanthine-xanthine oxidase system (74) as described previously (56).

Analyses of Metal Ion Sensitivity—Cells were grown for 48 h without shaking, re-inoculated to OD$_{600}$ of 0.5, and grown to an OD$_{600}$ of 1.0 ($-1 \times 10^7$) at 30 °C. Each cell culture was diluted ($-2 \times 10^6$) and incubated into 5 ml of Edinburgh minimal medium containing 50 $\mu$M CuSO$_4$ and further supplemented with increasing concentrations of metal ions specified in Fig. 8. After incubation for 7 days at 30 °C without shaking, total growth was measured at OD$_{600}$. 

**Fig. 2.** Transcription of pccs+ mRNA is unregulated by copper, silver, cadmium, and zinc, and a functional cuf1+ gene is not required for its expression. A, the isogenic strains FY254 (cuf1+) and SPY1 (cuf1Δ) were grown to mid-logarithmic phase in YES media. Cultures were incubated in the absence (−) or presence of CuSO$_4$ (100 and 1000 $\mu$M), or 100 $\mu$M BCS for 1 h at 30 °C. Total RNA was isolated and analyzed by Northern blot. pccs+ and act1+ mRNA steady-state levels are indicated by arrows. B, total RNA from the wild-type FY254 and the pccsΔ disruption strain were analyzed by RNase protection assay. pccs+ and act1+ mRNA levels are indicated by arrows. Total RNA was isolated from control untreated cells (−), and cells treated with CuSO$_4$ (100, 250, 500, and 1000 $\mu$M), AgNO$_3$ (100, 250, 500, and 1000 $\mu$M), CdCl$_2$ (100, 250, 500, and 1000 $\mu$M), or ZnSO$_4$ (100, 250, 500, and 1000 $\mu$M). The results shown are representative of three independent experiments.
The Fission Yeast Pccs and Copper

Fig. 3. Expression of full-length Pccs or Pccs domains I-III complements loss of CCS1 in S. cerevisiae. A, EGY103lys7Δ (also named EGY103ccs1Δ) fails to grow on synthetic complete medium lacking lysine in the presence of oxygen. EGY103ccs1Δ cells were transformed with plasmids expressing human CCS (hCCS) or S. pombe Pccs (pccs+) domains I-IV or domains I-III under the control of the S. cerevisiae CCS1 gene promoter and assayed for growth in the presence or absence of oxygen. As a positive control, EGY103ccs1Δ cells were also transformed with a plasmid expressing S. cerevisiae CCS1 (CCS1) under the control of its own promoter and assayed for growth in the same conditions. B, EGY103ccs1Δ displays a deficiency in SOD1 activity when cells are grown under copper starvation conditions in the presence of the Cu2+ chelator BCS (100 μM). These cells were transformed with vector only (−) or plasmids expressing the indicated domains of Pccs or CCS1 as positive controls. Total extracts from transformed cells were assayed for SOD1 activity using an in-gel activity assay with nitro blue tetrazolium staining. C, SOD activity was determined from the cell lysates used in B using a spectrophotometric method with cytochrome c and xanthine oxidase. The SOD activities reported represent the means of three replicates experiments ± S.D. D, Western blot analysis of extracts used in B employing an antibody directed against the S. cerevisiae SOD1 protein (89). Cellular levels of PGK was determined as a load control.

Results

The Primary Sequence of Pccs—Analysis of genomic DNA sequences from the S. pombe Genome Project revealed an open reading frame (SPAC22E12.04) related to two previously identified groups of copper homeostasis proteins: the CCS and MT proteins, which are involved in delivering copper to SOD1 and in copper detoxification, respectively. The SPAC22E12.04-encoded protein, which we have denoted Pccs, has four distinct domains. The first three domains of the protein have extended homology to the CCS proteins, including S. cerevisiae CCS (CCS1) and human CCS (hCCS) (Fig. 1). Domain I of Pccs (residues 1–187) displays 24 and 18% identity to the same region of the S. cerevisiae and human CCS proteins, respectively. Interestingly, domain I of Pccs lacks the Met-X-Cys-X2-Cys motif found in CCS1 and hCCS that is predicted to coordinate a single Cu2+ atom under conditions of copper starvation (39). Instead, Pccs has a single cysteine residue at position 11 that aligns with the last cysteine of the 2-Cys sequence (Fig. 1). Domain II of Pccs (residues 187–203) exhibits 28 and 23% identity with the domain II of S. cerevisiae CCS1 and human CCS, respectively. Supporting the notion that domain II of Pccs has greater similarity to CCS1 than hCCS, this domain lacks the three histidine and one aspartic acid residues that bind zinc in SOD1 and are preserved in human CCS (76). Furthermore, three of the four histidine residues known to bind copper in SOD1 and are conserved in hCCS domain II are absent in Pccs domain II. However, consistent with the model that domain II is involved in recognizing SOD1 and the subsequent formation of the CCS-SOD1 heterodimer, the residues predicted to be involved in protein-protein interactions between CCS and SOD1 (40) are highly conserved in all three Pccs, CCS1, and hCCS molecules (Fig. 1). Domain III of Pccs (residues 187–222) shows 47% identity with the domain III of both the S. cerevisiae and human CCS. This is the most conserved region, with an invariant Cys-X-Cys motif that is capable, in the case of CCS1, of binding copper (39). As shown in Fig. 1, the Pccs protein has an extra domain (residues 223–297) at its C terminus, which is not found in CCS1 or hCCS. The sequence of Pccs domain IV is

RNA Analysis—The S. pombe isogenic strains FY254 (wild type) and SPY1 (ccs1Δ) were grown in YES medium. Copper-treated (1 and 100 μM), BCS-treated (100 μM), and control cultures were grown to mid-logarithmic phase (OD600 of ~1.0). After a 1-h incubation at 30 °C total RNA was extracted by the hot phenol method (75). RNAs were quantitated spectrophotometrically, and 20 μg of RNA per sample were analyzed by Northern blot using random-primed 32P-labeled DNA probes. For RNase protection analyses (57), two plasmids were used to make antisense RNA probes. The plasmid pSKact1- was described previously (57). The plasmid pSKpccs- was constructed by inserting a 173-bp BamHI-EcoRI fragment of the pccs+ cDNA into the same sites in pBluescript SK. The antisense RNA hybridizes to the region between +172 and +346 upstream of the initiator codon of pccs+.
highly reminiscent of the metal binding protein, MT (77). This relatively short protein segment contains 17 cysteine residues, eight pairs of which are present in a Cys-Cys configuration. Furthermore, lysine and serine residues represent 19 and 28% of the total Pccs domain IV, respectively. Similar to MTs, domain IV lacks aromatic amino acids. Based on these observations and upon inspection of various sequence databases, the sequence of Pccs domain IV exhibits characteristics that are hallmarks of MTs. Therefore, Pccs bears strong similarity to domains found in the copper delivering CCS proteins, as well as the metal ion buffering MTs.

*pccs* mRNA Is Moderately Abundant in Wild-type Cells, Not Regulated by Metal Ion Repletion or Starvation, and Present in *cuflΔ* Cells—As determined by RNA blotting, the steady-state levels of *pccs* mRNA in wild-type strain FY254 are unaffected by either exogenous copper or the copper chelator BCS (Fig. 2A). Although the isogenic *cuflΔ* strain exhibited diminished levels of the *pccs* mRNA, no significant copper-dependent changes in *pccs* gene expression were observed (Fig. 2A). To further examine if *pccs* transcription is regulated by metal ions, the wild-type strain was grown in the presence of different metals at various concentrations, and the steady-state levels of *pccs* mRNA was assayed by RNase protection experiments. We tested the metal ions, Cu²⁺, Ag⁺, Cd²⁺, and Zn²⁺, and found no significant alteration in the transcription of *pccs* mRNA in response to these metals (Fig. 2B). Furthermore, our data indicate that although Cuf1 serves as a transcription factor that is required for expression of genes involved in copper transport, inactivation of the *cufl* locus does not affect the transcriptional competency of the *pccs* gene. Consistent with this observation, there were no changes in the *pccs* steady-state mRNA levels in response to either metal repletion or starvation.

Full-length Pccs or a Polypeptide HARBOURING the First Three Domains of Pccs Complements S. cerevisiae *ccs1*-null Phenotypes—To begin to understand the role of Pccs in copper homeostasis and to dissect the functions of its domains, we cloned two different alleles of *pccs*; one encoding the full-length Pccs protein, and the other encoding its first three domains. For this initial characterization, we expressed these *pccs* alleles under the regulation of the *S. cerevisiae* *CCS1* promoter in a *ccs1Δ* mutant strain of *S. cerevisiae*. In the presence of oxygen, the *ccs1Δ* mutant strain cannot grow on synthetic media without lysine because holo-SOD1 is required for aerobic lysine biosynthesis (78, 79). Transformation of this strain with plasmids expressing Pccs domains I-IV or Pccs domains I-III permitted cell growth under aerobic conditions (Fig. 3A). The growth was also restored when either *S. cerevisiae* *CCS1* or human CCS proteins were expressed in the *ccs1Δ* cells as shown previously (29) and reproduced here as controls (Fig. 3A). To characterize the ability of these alleles to activate SOD1, its activity was assayed in whole cell extracts from wild-type and *ccs1Δ* cells transformed with *pccs* I-IV and *pccs* I-III, grown under low basol copper conditions, using native enzyme polyacrylamide gel electrophoresis. The *ccs1Δ* strain, as observed previously (29), was devoid of detectable SOD1 activity, whereas *ccs1Δ* cells bearing the *pccs* I-IV or *pccs* I-III allele exhibited wild-type levels of SOD1 activity (Fig. 3B). In all cases, we also determined SOD activity in native cell extracts by measuring the inhibition of the reduction rate of cytochrome c by SOD, which competes for reactive oxygen species produced from the xanthine-xanthine oxidase system (Fig. 3C). This assay was conducted on the same cell lysates used for the in-gel staining assay (Fig. 3B). Importantly, the SOD activities measured by spectrophotometric analysis (Fig. 3C) very closely paralleled the results with the in-gel assay (Fig. 3B). When the *ccs1Δ* disruptant was transformed with the full-length

Pccs and Pccs domains I-III SOD1 activity levels were restored to 117 and 133%, respectively, compared with the levels in *ccs1Δ* cells harboring a wild-type copy of the *CCS1* gene expressed from a plasmid (Fig. 3C). To verify that the SOD1 protein was present in the wild-type and *ccs1Δ* cells, total protein extracts from cells transformed with plasmids expressing the indicated CCS molecules were analyzed by immunoblotting (Fig. 3D). These results showed that detectable levels of SOD1 were present in the *ccs1Δ* strain, indicating that the lack of activity in the *ccs1*-null strain was not due to lack of SOD1 expression. Taken together, these results demonstrate that the full-length *S. pombe* Pccs and Pccs domains I-III polypeptides can substitute for CCS1 in delivering copper to SOD1 in *S. cerevisiae* *ccs1Δ* cells.

![Fig. 4. An S. pombe pccsΔ strain displays a deficiency in SOD1 activity under copper-limiting conditions.](http://www.jbc.org/)

*Fig. 4.* An *S. pombe* pccsΔ strain displays a deficiency in SOD1 activity under copper-limiting conditions. *A*, SOD1 activity was determined in pccsΔ cells that were grown under conditions in which copper is required (100 μM BCS), in the presence of exogenous CuSO₄ (100 μM) and after transformation with a wild-type copy of the *pccs* gene expressed from a plasmid. *B*, aliquots of whole cell extracts used in *A* were analyzed by immunoblotting. The positions of the SOD1 and PCNA proteins are indicated with arrows. *C*, SOD1 activity was determined in a wild-type strain of *S. pombe* (WT) and an *S. pombe* sod1Δ and pccsΔ disruption strains in the presence or absence of exogenous CuSO₄ (100 μM). *D*, a plasmid-borne copy of the *sod1* gene was tested for its ability to restore SOD1 activity to a *S. pombe* strain with a deletion of the *sod1* gene. *E*, the indicated strains were incubated in the absence (−) or presence of CuSO₄ (100 μM) or BCS (100 μM). Protein extracts prepared from these strains were analyzed for steady-state protein levels of SOD1 by immunoblotting using either anti-SOD1 or anti-PCNA (as an internal control) antibody.
**Pccs Plays a Crucial Role in Activating S. pombe SOD1 under Conditions of Copper Starvation**—To further investigate the role of Pccs in *S. pombe*, the pccs" locus was insertionally inactivated by deletion and replacement with the *S. pombe* *ura4* gene or *kanMX6* genetic marker (80). An *S. pombe* strain bearing the disrupted pccsΔ allele displayed a deficiency in SOD1 activity (Fig. 4A). As expected, normal SOD1 activity could be rescued either by expressing a wild-type copy of the pccs" gene from a plasmid, or by the addition of CuSO₄ to the growth medium at a concentration of 100 μM (Fig. 4A). Importantly, Western blot analysis (Fig. 4B) of the same lysates shown in Fig. 4A revealed that the absence of SOD1 activity in the *pccsΔ* mutant strain was not due to lack of SOD1 expression. To assess the specificity of the non-denaturing gel electrophoresis and nitro blue tetrazolium staining for SOD1 activity, the sod1" gene was inactivated in the *S. pombe* strain FY254 to generate a sod1Δ strain. Cell lysates from this strain was analyzed for SOD1 activity. As shown in Fig. 4, C and D, sod1Δ mutant cells were defective in SOD1 activity in the absence or presence of copper. Furthermore, no SOD1 protein was detected in the sod1Δ mutant strain, unless a wild-type copy of the sod1" gene expressed from a plasmid was transformed into the cells (Fig. 4E). To determine if the first three domains of Pccs is sufficient to activate SOD1, the *pccsΔ* strain expressing a pccs" allele encoding domains I-III. The levels observed were comparable to those found in the same strain (*pccsΔ*) expressing full-length Pccs. In contrast, a *pccsΔ* mutant strain expressing the pccs" II-III-IV or pccs" II-III allele failed to activate SOD1 under copper-limiting conditions (Fig. 5B). This lack of superoxide scavenging activity does not reflect low protein levels, because the Pccs protein species were stably expressed in these cells (see supplemental data).² Moreover, a *S. cerevisiae ccs1Δ* strain expressing the pccs" II-III-IV allele displayed a deficiency in SOD1 activity and failed to grow on medium lacking lysine in the presence of oxygen (see supplemental data).³ These data suggest that the Pccs N-terminal domain I is required to activate SOD1 in conjunction with domains II and III under conditions of copper deprivation. Furthermore, these results suggest that Pccs functions in the same pathway as the *S. cerevisiae CCS1* protein in providing copper to SOD1.

Based on previous studies that determined ~95–99% of total *S. cerevisiae CCS1* resides in the cytosol (81, 82), we ascertained the localization of the Pccs protein in *S. pombe*. To ensure that insertion of GFP at the Pccs C terminus did not interfere with its function, we transformed the pccs" I-IV-GFP strain expressing a pccs" allele encoding domains I-III. The levels observed were comparable to those found in the same strain (*pccsΔ*) expressing full-length Pccs. In contrast, a *pccsΔ* mutant strain expressing the pccs" II-III-IV or pccs" II-III allele failed to activate SOD1 under copper-limiting conditions (Fig. 5B). This lack of superoxide scavenging activity does not reflect low protein levels, because the Pccs protein species were stably expressed in these cells (see supplemental data).² Moreover, a *S. cerevisiae ccs1Δ* strain expressing the pccs" II-III-IV allele displayed a deficiency in SOD1 activity and failed to grow on medium lacking lysine in the presence of oxygen (see supplemental data).³ These data suggest that the Pccs N-terminal domain I is required to activate SOD1 in conjunction with domains II and III under conditions of copper deprivation. Furthermore, these results suggest that Pccs functions in the same pathway as the *S. cerevisiae CCS1* protein in providing copper to SOD1.

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![Image](http://www.jbc.org/)

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² Gel nitro blue tetrazolium assay showing the SOD1 activity of Pccs-HA, derivative polypeptides is shown; a Western blot analysis of each polypeptide using an anti-HA antibody.

³ Spot test for complementation of aerobic lysine auxotrophy is shown; an analysis of SOD1 activity by the nitro blue tetrazolium gel assay.
plasmids containing the pccs genes functionally complemented the pccs mutant by 40%. The pccsΔ mutant strain displayed an increased sensitivity to copper toxicity, with 40% growth inhibition in the presence of 50 μM CuSO₄. To further investigate the ability of Pccs to confer protection against copper toxicity, we created a chimeric protein containing the first 222 amino acids (domains I-III) of Pccs fused to the residues 1-50 of Zym1. Expression of the chimeric Pccs-I-IV-Zym1 protein in the ace1Δ disruptant strain allowed these cells to grow in the presence of 500 μM CuSO₄. To study the effects of copper on the localization of Pccs, we used fluorescence microscopy to examine the localization of each GFP fusion in the wild-type strain. As shown in Fig. 6A, the full-length Pccs protein was predominantly localized in the cytosol, and in most cells, were largely excluded from the nucleus (Fig. 6 and data not shown). Our data do not allow us to establish whether or not a fraction of the total Pccs protein is localized in the mitochondrial intermembrane compartment as reported previously for CCS1 (81). Taken together, these observations suggest that the full-length Pccs and Pccs I-III proteins are primarily cytosolic components similar to SOD1.

Pccs Domain IV Confers Resistance to Copper When Expressed in an S. cerevisiae ace1Δ Strain—As mentioned above, analysis of the Pccs domain IV sequence revealed that this domain harbors a significant similarity to the MTs. As shown in Fig. 7A, several cysteine, serine, and lysine residues in Pccs domain IV align to those of the S. pombe Zym1 and human MT-I proteins. Interestingly, Pccs domain IV contains the conserved Lys²⁹⁴-X-Ser²⁴⁶ motif, which is invariant in the MT structure because of its role in joining the α- and β-domains of the protein (77, 83). Consistent with a role in metal ion sequestration, expression of the pccs-I-IV-encoded domain IV in S. cerevisiae cells that are hypersensitive to copper toxicity restored the growth of these cells in conditions of copper excess (Fig. 7B). To ascertain if functional restoration of copper tolerance in S. cerevisiae ace1Δ cells correlated specifically with the presence of Pccs domain IV, cells that were hypersensitive to copper were also transformed with the vector alone or the wild-type pccs-I-IV or pccs-I-III alleles. ace1Δ cells transformed with the vector alone exhibited no growth on medium supplemented with exogenous copper. Although ace1Δ mutant cells expressing the first three domains of Pccs prevented copper toxicity in the presence of 50 μM CuSO₄, these cells were sensitive to copper at concentrations of 100 or 500 μM CuSO₄ (Fig. 7B). Ectopic expression of the full-length pccs-I-IV gene in the S. cerevisiae ace1Δ strain in the presence of 50, 100, or 500 μM CuSO₄, allowed the transformed cells to grow in the presence of all elevated copper concentrations (Fig. 7B). Analogous to wild-type Pccs or Pccs domain IV, expression of the S. pombe Zym1 MT in S. cerevisiae ace1Δ cells, allowed these cells to grow in the presence of 500 μM CuSO₄. To further investigate the ability of Pccs to confer protection against copper toxicity, we created a chimeric protein containing the first 222 amino acids (domains I-III) of Pccs fused to the residues 1-50 of Zym1. Expression of the chimeric Pccs-I-IV-Zym1 protein in the ace1Δ disruptant strain allowed these cells to grow when copper was present in excess of physiological requirements, at the same rate as cells expressing the wild-type Pccs protein (Fig. 7B). Taken together, these results show that the Pccs protein through its domain IV plays a critical role in cell survival under conditions of copper toxicity.
pccs and zym1Δ slightly increased the sensitivity to cadmium compared with the pccsΔ single mutant (Fig. 8B). Thus, when environmental metal ion levels are elevated, the Pccs protein appears to play a physiological function in protecting fission yeast cells against both copper and cadmium toxicity.

**Overexpression of Pccs Domain IV Suppresses the Copper Toxicity Phenotype Resulting from ctr6Δ**—Based on the findings that deletion of the pccsΔ gene lowered copper tolerance in *S. pombe* and that both Pccs and Pccs domain IV conferred copper resistance when expressed in *S. cerevisiae ace1Δ* cells, we ascertained the ability of these proteins to elicit copper resistance when overexpressed in an *S. pombe* strain that is hypersensitive to copper. Overexpression of ctr6Δ from the thiamine-regulated nmt1Δ promoter resulted in an increased sensitivity to copper toxicity when transformed cells were grown on medium containing elevated concentrations of CuSO4 (56). Cotransformation of both the nmt1-ctr6Δ and nmt1-pccsΔ I-IV genes into *S. pombe* cells conferred resistance to 15 μM copper on the transformed cells compared with the same cells that were co-transformed with nmt1-ctr6Δ and an empty vector (pREP3X) (Fig. 9). Similar to the expression of the full-length pccsΔ gene, cells expressing the pccsΔ IV allele displayed no copper sensitivity. On the other hand, cells expressing the pccsΔ I-II-III allele (without domain IV), were sensitive to copper. Consistent with its ability to sequester divalent metal ions, expression of zym1Δ conferred copper tolerance to the transformed cells. Furthermore, coexpression of the chimeric 1Pccs222-1Zym150 and Ctr6 proteins in the ctr6Δ pccsΔ zym1Δ mutant strain allowed these cells to grow in the presence of exogenous copper at the same level as cells expressing the wild-type Pccs protein. This finding indicates that the first three domains of Pccs can be fused to a MT-like polypeptide to allow detoxification of excess copper ions. Together with the results from our studies on the *ace1Δ* yeast strain, these data strongly indicate that the role of Pccs in copper buffering is mediated through its fourth domain.

**DISCUSSION**

In eukaryotes, the CCS proteins function in the delivery of copper to the SOD1 enzyme (31, 84). The copper- and zinc-containing SOD is a free radical detoxifying protein that catalyzes the disproportionation of superoxide ion to yield hydrogen peroxide and dioxygen (85). Genetic, biochemical, and structural data have demonstrated fundamental features of CCS chaperones that revealed a mode of action for both the binding of copper and its specific requirement to incorporate copper into SOD1 (86). Typically, three distinct domains compose the copper carrier CCS (39). Domain I at the N terminus functions in copper binding under conditions of copper deprivation. Domain
II is required for appropriate docking between CCS and SOD1. Domain III, which is highly conserved among CCS molecules across different species, binds copper and participates in the interaction with SOD1 during the process of copper transfer (39). The existence of a gene in fission yeast that encodes a CCS molecule with an extra domain at the C terminus of the protein raises two important questions: (i) What is the role of the fourth domain with respect to the function of the copper chaperone? (ii) What is the specific contribution of domain IV to cellular metal ion homeostasis in *S. pombe* cells? To address these questions, we first utilized a *S. cerevisiae* yeast system (70) in which the endogenous *CCS1* gene was inactivated. By using this approach, we sought to ensure the presence of Pccs as the sole protein with the ability to activate SOD1. When expressed in *S. cerevisiae ccs1/H9004* cells under conditions of copper scarcity, the full-length *S. pombe* Pccs or a Pccs polypeptide spanning domains I to III provided cells with robust levels of activated SOD1. Furthermore, both forms of Pccs complemented the lysine auxotrophy of the *ccs1/H9004* mutant cells. Therefore, these data revealed that both full-length Pccs and Pccs domains I-III are sufficient for delivering copper to SOD1. These data further imply that the C-terminal domain IV of Pccs is dispensable with respect to the activation of SOD1 in vivo. To ascertain if the presence of the first three domains of Pccs was sufficient for SOD1 activity in *S. pombe*, the chromosomal *pccs/H11001* gene was deleted by homologous recombination and SOD1 activity evaluated. Deletion of *pccs/H11001* gene resulted in the loss of SOD1 activity under copper-limiting conditions. Similar to the *S. cerevisiae ccs1Δ* cells, the expression of only the first three domains of Pccs was sufficient to allow *pccs/H9004* mutant cells to restore SOD1 activity. Moreover, a *S. pombe pccsΔ* strain expressing the *S. cerevisiae CCS1* gene was also capable of restoring SOD1 activity when copper was limiting. 4 On the basis of these data, we conclude that the presence of the first

4 J. Laliberté and S. Labbé, unpublished data.
three domains of Pccs is sufficient for normal SOD1 activity in both yeast species.

With respect to the second question, the analysis of Pccs function in a S. cerevisiae ace1Δ strain revealed that expression of Pccs domain IV conferred copper tolerance to these cells, protecting them from lethal copper concentrations. Similarly, ace1Δ cells expressing the full-length Pccs or Zym1 MT protein acquired copper tolerance. Furthermore, consistent with the hypothesis that Pccs participates in intracellular copper buffering mainly through its fourth domain, we showed that ace1Δ cells expressing the first three domains of Pccs are more sensitive to copper by an order of magnitude compared with cells expressing full-length Pccs or Pccs domain IV. The involvement of Pccs in metal ion tolerance was further supported by three additional observations. First, the analysis of a S. pombe strain in which pccs+ has been insertionally inactivated clearly demonstrates a phenotype with marked sensitivity to copper and cadmium ions. Second, the primary sequence of Pccs domain IV exhibits extensive homology to MTs and specifically harbors numerous repeats of a putative copper-binding Cys–Cys motif. Third, in a S. pombe strain overexpressing the mnt11–crt6 allele that generates a hypersensitivity to copper ions, overexpression of Pccs domain IV, as well as full-length Pccs, protects the cells from the toxic effects of copper ions. Taken together, these data suggest a model wherein the first three domains of Pccs are required to specifically deliver copper to SOD1 under low copper concentrations, while the fourth domain of Pccs functions to sequester metals in the presence of elevated copper concentrations.

In this study, we inactivated the sod1+ locus by deletion and replacement with the S. pombe urad4+ gene. Although the sod1Δ cells grow poorly in shaken and well-aerated cultures, these cells were viable on standard YES medium or in a modified Edinburgh medium containing supplements of cysteine, methionine, lysine, adenine, histidine, and leucine. It should be noted that recent studies of S. pombe with a disrupted sod1+ gene showed that the viability of sod1Δ mutants varied depending on the genetic background of the fission yeast strain (87, 88). Although the nature of this genetic variation is not clear, it is possible that the composition of the growth media may be a factor due to the amino acid auxotrophies of sod1Δ cells.

Our results thus far support a dual role for Pccs in copper homeostasis. It functions as a copper chaperone when copper concentrations are limiting and as a detoxifier when copper concentrations are in excess. Because its function is required in both high and low levels of copper, it is not surprising that the steady-state levels of pccs+ mRNA is constitutive and unaffected by changes in copper concentrations. Consistently, expression of pccs+ mRNA at steady-state levels, was independent of cuf1+, a gene encoding the nutritional copper sensing trans-inducer of the copper transport genes ctnr4+, ctnr5+, and ctnr6+ in fission yeast. Interestingly, pccs+ mRNA was fairly abundant in wild-type S. pombe cells possibly to maintain adequate intracellular levels of Pccs for both copper distribution and detoxification pathways.

Two notable differences exist between the S. pombe and S. cerevisiae CCS chaperones. First, the N-terminal domain I of the S. pombe Pccs lacks the copper-binding Met-X-Cys-X2-Cys motif, except for the last cysteine residue, and second, Pccs contains a fourth domain at its C terminus. Given that Pccs lacking the N-terminal domain I cannot activate the SOD1 under copper-limiting conditions, it is likely that other residues within domain I besides the Met-X-Cys-X2-Cys site that is found in CCS1 but not Pccs are important for its metallochaperone-like activity in vitro. Efforts are currently underway to identify the residues within domain I that may be important for this function. Based on computer algorithm analysis, the C-terminal 75 amino acids of Pccs exhibited sequence similarity to MTs. Thus far, a single MT encoded by the zym1+ locus has been described in S. pombe (51). In fission yeast cells, zym1+ is transcriptionally induced by zinc and cadmium, but not copper (51). Although Zym1 suppresses zinc toxicity (51), our findings showed that S. cerevisiae ace1Δ cells expressing Zym1 were protected against copper toxicity. Deletion of zym1+ (zym1Δ) from fission yeast resulted in reduced copper tolerance in the mutant strain. However, as reported previously (51), the steady-state levels of zym1+ mRNA were unaffected by cellular copper status. Interestingly, deletion in the pccs+ (pccsΔ) in S. pombe cells also resulted in increased sensitivity to copper toxicity. In a heterologous strain, expression of pccs+I-IV or pccs+IV in the copper sensitive S. cerevisiae ace1Δ cells conferred significant levels of resistance to these cells. Using this strain, we further demonstrated that a chimeric Pccs protein harboring the S. pombe metallothionein Zym1 instead of Pccs domain IV allowed detoxification of excess copper at the same level as the wild-type protein. Thus, the fact that there is no known MT in S. pombe whose expression is induced by the presence of copper, fission yeast cells may circumvent this situation by the use of two genes, pccs+ and zym1+, to protect cells against copper poisoning.

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The *Schizosaccharomyces pombe* Pccs Protein Functions in Both Copper Trafficking and Metal Detoxification Pathways

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