Functional dissection of Ctr4 and Ctr5 amino-terminal regions reveals motifs with redundant roles in copper transport

Jude Beaudoin, Julie Laliberté and Simon Labbé

Correspondence

Simon Labbé

Simon.Labbe@USherbrooke.ca

Département de Biochimie, Faculté de Médecine, Université de Sherbrooke, 3001 12e Ave Nord, Sherbrooke, QC, Canada J1H 5N4

Copper uptake in the fission yeast *Schizosaccharomyces pombe* is carried out by a heteromeric complex formed by two proteins, Ctr4 and Ctr5. In this study, a stable expression system using integrative plasmids was developed to investigate the respective roles of Ctr4 and Ctr5 in copper transport. It was shown that expression of full-length Ctr4 or truncated Ctr4 containing residues 106–289 was required for localization of Ctr5 to the plasma membrane. Likewise, when the full-length Ctr5 or truncated Ctr5 from residues 44–173 was co-expressed with Ctr4, this protein was visualized at the periphery of the cell. To determine the importance of the Mets motifs (consisting of five methionines arranged as Met-X₂-Met-X-Met, where X is any amino acid) of Ctr4 and Ctr5 in the heteroprotein complex, we co-expressed Ctr5 lacking the Mets motif and Cys-X-Met-X-Met sequence with wild-type Ctr4 or its mutant derivatives. Conversely, Ctr4 lacking the Mets motif and Met¹²² was expressed with wild-type Ctr5 or its mutant derivatives. These experiments revealed that the five Mets motifs of Ctr4 and the Ctr4 residue Met¹²² have equally important roles in copper assimilation. Furthermore, the two partially overlapping Mets motifs and the Cys-X-Met-X-Met sequence in Ctr5 have redundant functions in copper transport, with the latter sequence making a greater contribution than the former. Together, the data reveal that co-expression of both Ctr4 and Ctr5 is necessary for the proper function and localization of the heteroprotein complex to the plasma membrane. Once on the cell surface, the N-terminal regions of Ctr4 and Ctr5 can function independently to transport copper; however, the greatest efficiency is achieved when both N termini are present.

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INTRODUCTION

All living organisms, from bacteria to humans, require copper (reviewed by Rees & Thiele, 2004a). Because of the ease with which it can gain and lose electrons, copper serves as an electron-transfer intermediate for a variety of cellular enzymes (reviewed by Peña *et al.*, 1999). However, when present in excess, copper becomes toxic, reacting with hydrogen peroxide or dioxygen to produce hydroxyl radicals that can damage DNA, proteins and membrane lipids (Halliwell & Gutteridge, 1984). A critical balance must therefore be maintained by specialized cellular transport mechanisms to regulate intracellular copper content.

Biological management of copper requires uptake from the environment through the cellular membrane for delivery to

copper-containing enzymes (reviewed by Puig & Thiele, 2002a). Studies in the baker's yeast *Saccharomyces cerevisiae* have identified genes encoding components of the high-affinity copper-transport machinery that resides at the cell surface. Extracellular Cu²⁺ is reduced by the Fre1 and Fre2 metalloreductases (Hassett & Kosman, 1995; Georgatsou *et al.*, 1997; Martins *et al.*, 1998). Either subsequent to, or concomitant with, reduction, Cu⁺ is taken up through two high-affinity copper transporters, Ctr1 (Dancis *et al.*, 1994a, b; Puig *et al.*, 2002b) and Ctr3 (Knight *et al.*, 1996; Peña *et al.*, 2000). Although Ctr1 and Ctr3 are functionally redundant, these two plasma-membrane proteins mediate copper uptake independently of each other (Peña *et al.*, 2000). Based on bioinformatics and biochemical analyses, Ctr1 and Ctr3 possess three predicted transmembrane domains (reviewed by Puig & Thiele, 2002a). The N terminus of Ctr1 harbours eight copies of the sequence Met-X₂-Met-X-Met, called the Mets motif (Dancis *et al.*, 1994a; Puig *et al.*, 2002b). The Ctr1 Mets motifs are exposed to the extracellular face of the plasma membrane (Puig *et al.*, 2002b). Although the eight Mets motifs present in Ctr1 play an important role in copper assimilation when cells are grown under copper-deficient conditions, the last

Abbreviations: BCS, bathocuproine disulphonic acid; GFP, green fluorescent protein; YES, yeast extract plus supplements.

Supplementary figures showing the fluorescence microscopy visualization of the cellular location of wild-type or mutant Ctr4–GFP proteins, and the visualization of wild-type or mutant Ctr5–MYC₁₂ proteins by indirect immunofluorescence microscopy, are available with the online version of this paper.

methionine (Met¹²⁷) of the eighth Mets motif is essential for Ctr1 function (Puig *et al.*, 2002b). Likewise, a Met-X₃-Met motif (residues 256–260) within the second transmembrane domain of Ctr1 has also been shown to be indispensable for the function of Ctr1 in high-affinity copper uptake (Puig *et al.*, 2002b). Despite the fact that the Ctr3 protein exhibits a limited overall sequence homology to Ctr1, it has been shown that Ctr3 contains a similar Met-X₃-Met motif (residues 185–189) within its second transmembrane domain (Puig *et al.*, 2002b). This enables Ctr3 to import copper from the plasma membrane, in conjunction with other critical residues, such as Cys¹⁶ within the N-terminal region, Cys⁴⁸ and Cys⁵¹ within the first transmembrane domain, and Cys¹⁹⁹ within the third transmembrane domain (Peña *et al.*, 2000).

When environmental copper levels are scarce, the early molecular mechanisms of copper capture in the fission yeast *Schizosaccharomyces pombe* differ somewhat from those of *Sac. cerevisiae*. While deletion of the *ctr4*⁺ gene by homologous recombination in fission yeast abrogates high-affinity copper transport (Labbé *et al.*, 1999), its heterologous expression in a *ctr1Δ ctr3Δ Sac. cerevisiae* strain is insufficient to complement the copper-transport defect in this strain (Zhou & Thiele, 2001). Complementation requires a second *S. pombe* gene, *ctr5*⁺ (Zhou & Thiele, 2001). In the absence of Ctr5, a Ctr4–GFP (green fluorescent protein) fusion protein is mislocalized to an intracellular compartment likely to be the endoplasmic reticulum (Zhou & Thiele, 2001). However, when co-expressed with Ctr5 in *ctr1Δ ctr3Δ Sac. cerevisiae* cells, Ctr4–GFP is localized to the plasma membrane (Zhou & Thiele, 2001). Likewise, when functional Ctr4–GFP and Ctr5–GFP fusion proteins are co-expressed in *S. pombe* cells from high-copy-number episomal plasmids, both proteins are localized in the plasma membrane. However, in the absence of Ctr5–GFP, the bulk of Ctr4–GFP fusion protein is found in a perinuclear compartment (Zhou & Thiele, 2001). Co-immunoprecipitation experiments reveal that the Ctr4 and Ctr5 proteins are found in a complex at the membrane (Zhou & Thiele, 2001). However, within this heterocomplex, the exact role of each protein remains unexplored. We have observed that both proteins have N-terminal conserved sequences that could serve as potential copper ligands. This suggests that both Ctr4 and Ctr5 may bind copper through these sequences, and that this may be an important aspect of their respective functions in copper transport. This study presents the first molecular dissection of the fission-yeast two-component copper-transporting system, which comprises the Ctr4 and Ctr5 proteins. The data reveal functional N-terminal domains whereby the heteroprotein complex transports copper as a function of copper availability.

METHODS

Yeast strains and media. *S. pombe* strains used in this work were the wild-type FY435 (*h*⁺ *his7-366 leu1-32 ura4-Δ18 ade6-M210*) (Bezanilla *et al.*, 1997), and the *ctr4Δ* (isogenic to FY435 plus

ctr4Δ::ura4⁺), *ctr5Δ* (isogenic to FY435 plus *ctr5Δ::KAN*^r) and *ctr4Δ ctr5Δ* double-mutant (isogenic to FY435 plus *ctr4Δ::ura4*⁺ *ctr5Δ::KAN*^r) disruption strains. Yeast strains were grown at 30 °C in yeast extract plus supplements (YES) or in selective Edinburgh minimal medium (EMM) supplemented with the appropriate auxotrophic requirements (Alfa *et al.*, 1993). The respiratory carbon source medium YES/glycerol/ethanol (glycerol/EtOH) was prepared by replacing the glucose in YES with 3 % glycerol (v/v) and 2 % ethanol. Growth under conditions of low copper availability was carried out as described previously (Beaudoin *et al.*, 2003), except that the cells were grown to OD₆₀₀ ~1.0 and then treated with 100 μM of the copper chelator bathocuproine disulphonic acid (BCS) for 3 h instead of 1 h.

Ctr4 plasmids. To facilitate the construction of *ctr4* alleles, the plasmid pBluescript SK (Stratagene) was digested with *AccI*, treated with Klenow enzyme, and ligated to eliminate the *AccI* restriction site within the polylinker. The resulting plasmid was denoted pSKΔ*AccI*. An *SmaI*–*BspEI* *ctr4*⁺ promoter fragment up to –737 from the start codon of the *ctr4*⁺ gene was isolated by PCR. *BspEI*–*BamHI* fragments of the wild-type and *ctr4*⁺–GFP fusion alleles were isolated by PCR from the pSP*ctr4*⁺B-E/1.6 and pSP*ctr4*⁺GFP plasmids (Labbé *et al.*, 1999), respectively. Plasmids pSK-737*ctr4*⁺ and pSK-737*ctr4*⁺–GFP were constructed via three-piece ligation by simultaneously introducing the *SmaI*–*BspEI* *ctr4*⁺ promoter fragment and the *BspEI*–*BamHI* fragment from pSP*ctr4*⁺B-E/1.6 and pSP*ctr4*⁺GFP, respectively, into the *SmaI*/BamHI-cut pSKΔ*AccI* vector. The integrative fission-yeast plasmid pBP*ade6*⁺ was constructed by three-piece ligation by simultaneously introducing a 1727 bp *Asp718*–*HindIII* PCR-amplified fragment containing the *ade6*⁺ locus starting at –888 from the translational start codon up to +839 after the initiator codon, and a 1140 bp *HindIII*–*BamHI* PCR-amplified fragment containing the *ade6*⁺ locus starting at +840 to +1979, into the *Asp718*/BamHI-digested pBluescript SK vector (Stratagene). One primer was engineered to ensure the presence of the *BsiWI* restriction site at the end of the *ade6*⁺ locus. Subsequently, the pSK*ade6*⁺ plasmid was digested with *Asp718*, filled-in with Klenow, and digested with *BsiWI*. The purified *Asp718* (Klenow)–*BsiWI* DNA fragment was inserted into the *AadII* (which was blunt-ended by Mung Bean nuclease)/*BsiWI*-cut pJK148 plasmid (Keeney & Boeke, 1994). The resulting plasmid, named pBP*ade6*⁺, generated a useful *ade6*⁺ auxotrophic marker in place of a *leu1*⁺ marker for targeted integration at the *ade6*⁺ locus of *S. pombe*. The integrative plasmids pBP*ctr4*⁺ and pBP*ctr4*⁺–GFP were constructed by subcloning the *PstI*–*SpeI* fragments from pSK-737*ctr4*⁺ and pSK-737*ctr4*⁺–GFP, containing the entire *ctr4*⁺ and *ctr4*⁺–GFP genes, respectively, into the corresponding sites of pBP*ade6*⁺. The resulting plasmids were then used to integrate, in single copy, the wild-type and *ctr4*⁺–GFP genes at the *ade6* locus of the *ctr4Δ ctr5Δ* strain. By using this strategy, we ensured that the *ctr4*⁺ and *ctr4*⁺–GFP genes were under the control of the *ctr4*⁺ promoter. To generate the mutation of Met¹²² to Ala in the *ctr4*⁺ gene, the primer CTR4M122A (5′-CATCGATAGTATACCAGTTC-CAATACGCGGATAATTTACAAGAGAAGGCC-3′) was made with mutations (underlined). Furthermore, the primer was designed to ensure the presence of the *AccI* restriction site found naturally within the *ctr4*⁺ gene. Using a second primer that hybridized downstream at the *SmaI* restriction site, a *SmaI*–*AccI* fragment was amplified by PCR and then swapped for an identical DNA region into the pSK-737*ctr4*⁺ or pSK-737*ctr4*⁺–GFP plasmid. These plasmids were digested with *PstI* and *SpeI* and cloned into the *PstI*/SpeI-cut pBP*ade6*⁺ vector to generate pBP*ctr4M122A* and pBP*ctr4M122A*–GFP, respectively. Using either pBP*ctr4*⁺–GFP or pBP*ctr4M122A*–GFP, a set of deletions (Δ53, Δ58, Δ69, Δ81 and Δ93) was created within the N-terminal portion of Ctr4. Five primers containing a *BspEI* site were engineered to anneal pairwise to DNA at codons 54, 59, 70, 82 and 94 of *ctr4*⁺, respectively. The antisense primer for

amplification contained a *Bam*HI restriction site. The DNA sequence from each respective PCR-amplified fragment was digested with *Bsp*EI and *Bam*HI and exchanged with a DNA region into the pBPctr4⁺-GFP or pBPctr4M122A-GFP plasmid. To generate the ctr4-M6 mutant allele, the primer CTR4-M6-A (5'-GCACGCTCCGGAATGTCCAACAGCACAACATCCGCTCTGGCGCGAATGC-GACTAATACC-3') was made (underlined letters represent nucleotide substitutions that gave rise to mutations in the last Mets motif). This primer was used in conjunction with an antisense primer that hybridized immediately after the stop codon and in which a *Bam*HI restriction site was present. Once generated, the ctr4-M6 mutant allele was used to replace the equivalent DNA segment in pBPΔ93ctr4M122A-GFP. Plasmids pBPΔ105ctr4-GFP, pBPΔ135ctr4-GFP and pBPΔ166ctr4-GFP were created as follows: three PCR fragments encompassing the ctr4⁺-GFP ORF starting at +316, +406 and +499 from the initiator codon up to the stop codon, including the *gfp* tag, were amplified from pSK-737ctr4⁺-GFP. This was performed using primers designed to introduce *Bsp*EI and *Spe*I at the termini of the upstream and downstream DNA fragments, respectively. The PCR products obtained were digested with *Bsp*EI and *Spe*I and cloned into the corresponding sites of pBPctr4⁺-GFP.

Ctr5 plasmids. To generate the pSKctr5⁺ plasmid, a 1342 bp *Pst*I-*Sma*I PCR-amplified DNA segment containing the *S. pombe* ctr5⁺ locus starting at -820 from the translational start codon up to the stop codon was inserted into the *Pst*I and *Sma*I sites of pBluescript SK (Stratagene). To create a plasmid that has the ctr5⁺ gene with twelve copies of the *myc* epitope, the DNA fragment containing the ctr5⁺ gene and its promoter region was isolated from the pSKctr5⁺ plasmid using *Pst*I and *Sma*I and inserted into the corresponding sites of pctr4⁺-X-*myc*₁₂ (Bellemare *et al.*, 2001). By substituting the ctr5⁺ promoter in place of the ctr4⁺ promoter, the plasmid has the ctr5⁺ promoter driving the expression of the ctr5⁺-*myc*₁₂ fusion gene. Once created, the ctr5⁺-*myc*₁₂ fusion gene and its regulatory region were isolated using *Pst*I and *Xba*I and inserted into the corresponding sites of pJK148. The resulting integrative plasmid was designated pJKctr5⁺-*myc*₁₂. The ctr5 mutant alleles containing either site-specific mutations (*M1*, *M2*, *M3*, *M4*, *M5* and *M6*) or N-terminal deletions (pJKΔ43ctr5-*myc*₁₂ and pJKΔ76ctr5-*myc*₁₂) were created by the overlap-extension method (Ho *et al.*, 1989). The

DNA sequence of the *Pst*I-*Xba*I fragment from each respective PCR-amplified fragment was used to replace the corresponding fragment from plasmid pJKctr5⁺-*myc*₁₂. The DNA sequence of the *Pst*I-*Xba*I fragment from each respective mutant was confirmed by dideoxy sequencing.

Microscopic analysis of Ctr4 and Ctr5 localization. *ctr4Δ* *ctr5Δ* mutant cells expressing the GFP fusion proteins were grown in YES medium to OD₆₀₀ ~1.0. After incubation in the presence of 100 μM of the copper chelator BCS for 3 h, direct fluorescence microscopy was used to detect the Ctr4-GFP fusion protein or its mutant derivatives in live cells mounted in 1% low-melt agarose. Indirect immunofluorescence microscopy was used to localize the MYC₁₂ epitope-tagged versions of the Ctr5 protein. The cells were treated identically to those described above, except that after 3 h incubation in the presence of BCS (100 μM), the cells were fixed by adding formaldehyde (methanol-free) (Polysciences) to a final concentration of 3-7%. Fixed cells were harvested and washed with 0.1 M potassium phosphate, pH 6.5, containing 1.2 M sorbitol. Cells were spheroplasted as described previously (Bellemare *et al.*, 2002) and adsorbed to poly-lysine-coated multiwell slides. After a 30 min block with TBS (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% BSA), cells were incubated with anti-*c-myc* antibody (9E10) (Roche Diagnostics) diluted 1:200 in TBS. After an 18 h reaction, cells were washed with TBS and incubated for 4 h with goat anti-mouse Alexa Fluor 546 conjugate (Invitrogen) diluted 1:200 in TBS. The cells were viewed with a Nikon Eclipse E800 epifluorescent microscope equipped with a Hamamatsu ORCA-ER cooled charge-coupled device (CCD) camera.

RESULTS

Reciprocal effects of Ctr4 on Ctr5 localization and Ctr5 on Ctr4 localization

In *S. pombe*, the Ctr4-Ctr5 high-affinity copper-transport system consists of two structurally related transmembrane proteins (Fig. 1). An earlier study has shown that *S. pombe*

Fig. 1. Primary structural features of the Ctr4 and Ctr5 proteins. The top panel shows a schematic representation of the Ctr4 protein. The grey-shaded regions indicate the location of the five potential copper-binding motifs, termed Mets motifs (M-X₂-M-X-M). The position of Met¹²² in Ctr4 is indicated. The bottom panel shows a schematic representation of the primary structure of the Ctr5 protein. Ctr5 has two copies of partially overlapping Mets motifs and a C-X-M-X-M sequence, which lies downstream of the Mets motifs. The predicted transmembrane domains (TM1-3) of both proteins are shown as black rectangles. The amino acid sequence numbers refer to the position relative to the first amino acid of each protein.

strains harbouring a deletion of either *ctr4*⁺ or *ctr5*⁺ are defective in copper uptake (Zhou & Thiele, 2001). Consequently, these strains cannot grow on respiratory carbon sources such as glycerol/ethanol, presumably due to the lack of copper incorporation into mitochondrial cytochrome *c* oxidase (Zhou & Thiele, 2001). Because the earlier study was performed using single-deletion strains and genes expressed

from plasmids present in multiple copies, we sought to examine the requirement of Ctr5 for Ctr4 activity and *vice versa* using a double-disruption strain, *ctr4Δ ctr5Δ*, in which *ctr4*⁺ or *ctr5*⁺, or both genes, were returned in a low copy number by integration. As shown in Fig. 2(A), when both *ctr4*⁺ and *ctr5*⁺ were insertionally inactivated, the double-mutant strain failed to grow on respiratory carbon sources.

Fig. 2. Co-expression of both the Ctr4 and the Ctr5 protein is necessary for growth on respiratory carbon sources and for localization to the plasma membrane. (A) *S. pombe* cells harbouring a *ctr4Δ ctr5Δ* double deletion were transformed with empty integrative vectors (–), a vector alone and *ctr4*⁺, a vector alone and *ctr5*⁺, or *ctr4*⁺ and *ctr5*⁺. Cultures were spotted onto YES agar media containing glucose or glycerol/ethanol (EtOH) with 0 and 15 μM CuSO₄. WT, isogenic wild-type strain FY435. (B) Ctr4–GFP and Ctr5–MYC₁₂ fusion proteins are functional. The tagged proteins were expressed into a *ctr4Δ ctr5Δ* mutant strain as in (A) and tested for their ability to confer growth on respiratory carbon sources (glycerol/ethanol). (C) Localization of Ctr4–GFP and Ctr5–MYC₁₂ fusion proteins. Yeast cells disrupted for *ctr4*⁺ and *ctr5*⁺ that expressed individually, or in combination, the tagged genes were grown in YES medium containing 100 μM BCS and analysed by fluorescence microscopy. Cell morphology was examined through Nomarski optics.

This growth defect could be reversed by the addition of exogenous copper to the growth medium at concentrations of at least 15 μ M (Fig. 2A). Expression of *ctr4*⁺ in *ctr4* Δ *ctr5* Δ cells under the control of its native promoter was not sufficient to restore respiratory growth. Likewise, expression of *ctr5*⁺ in *ctr4* Δ *ctr5* Δ cells did not correct the respiratory growth defect. In contrast, co-expression of both *ctr4*⁺ and *ctr5*⁺ restored respiratory proficiency to these cells (Fig. 2A).

To determine the localization of Ctr4 and Ctr5, integrative plasmids harbouring wild-type copies of *ctr4*⁺ and *ctr5*⁺ were engineered with different epitopes at their C termini. A single copy of the gene encoding GFP was inserted downstream of and in-frame with the *ctr4*⁺ gene. Furthermore, twelve copies of the c-myc epitope were fused downstream of and in-frame with the *ctr5*⁺ coding region. Integrative plasmids expressing the tagged Ctr4 and Ctr5 were separately transformed or co-transformed into a *ctr4* Δ *ctr5* Δ strain, and each transformant was analysed for respiratory proficiency compared to the wild-type strain. As shown in Fig. 2(A, B), when Ctr5-MYC₁₂ and Ctr4-GFP were co-expressed in *ctr4* Δ *ctr5* Δ cells, they functionally complemented the respiratory deficiency of this strain at the same level as the Ctr4 and Ctr5 wild-type, untagged proteins. Furthermore, co-expression of the Ctr4-GFP and Ctr5-MYC₁₂ fusion proteins allowed their detection in the plasma membrane (Fig. 2C). In contrast, when Ctr4-GFP was expressed in the absence of Ctr5-MYC₁₂, Ctr4-GFP was largely trapped in a perinuclear structure that might correspond to an early secretory compartment (Fig. 2C). Likewise, when the Ctr5-MYC₁₂ fusion protein was expressed in the absence of Ctr4-GFP, Ctr5-MYC₁₂ was found in a perinuclear ringed structure, consistent with localization to an early secretory compartment (Fig. 2C). Taken together, these results reveal that, when expressed from integrated loci under their native promoters, Ctr4-GFP and Ctr5-MYC₁₂ are both essential for proper localization at the plasma membrane. Furthermore, both proteins are required for growth on respiratory carbon sources.

The N-terminal regions of both Ctr4 and Ctr5 play an important role in copper assimilation

Although co-expression of Ctr4 and Ctr5 restores copper acquisition to an *S. pombe* *ctr4* Δ *ctr5* Δ strain, the exact function of each protein within this heterocomplex at the plasma membrane is currently unclear. As shown in Fig. 1, the N termini of both proteins have conserved Mets motifs that are predicted to extend into the extracellular environment. We hypothesized that both Ctr4 and Ctr5 utilize these motifs to acquire copper from the environment, making them essential components of the copper-transport complex. To test this hypothesis, we used site-directed mutagenesis to convert each individual Met and Cys residue to Ala within the two Mets motifs and the Cys-X-Met-X-Met sequence of Ctr5-MYC₁₂ (Fig. 3A). This mutant allele was denoted *ctr5*-M6. The importance of the Ctr4 Met-rich sequences for Ctr4-Ctr5 function was assessed by

co-expressing the wild-type *ctr4*⁺-GFP and *ctr5*-M6 alleles in the *ctr4* Δ *ctr5* Δ (JSY22) strain and assaying for their ability to restore respiratory proficiency. This was compared to the parental strain FY435 or the *ctr4* Δ *ctr5* Δ mutant strain co-expressing *ctr4*⁺-GFP and *ctr5*⁺-myc₁₂. As shown in Fig. 3(A), the growth of cells co-expressing Ctr4-GFP and Ctr5-M6 on glycerol/ethanol medium was equivalent to cells expressing wild-type Ctr4-GFP and Ctr5-MYC₁₂, except when extracellular bioavailable copper was chelated by the addition of 20 or 25 μ M BCS. Under these conditions of copper deprivation, no growth was observed.

To assess the role of the two partially overlapping Mets motifs and a similar Cys-X-Met-X-Met sequence in Ctr5, mutant *ctr4*-M6 was constructed, in which the first four Mets motifs were deleted and the fifth Mets motif and Met¹²² were mutated to Ala. This mutant was co-transformed with wild-type *ctr5*⁺ into the *S. pombe* strain JSY22. As observed for cells co-expressing *ctr4*⁺-GFP with *ctr5*-M6, these cells were capable of growing on glycerol/ethanol medium with 0, 5 and 10 μ M BCS, but not in the presence of 20 or 25 μ M BCS (Fig. 3A). Furthermore, co-expression of the mutant proteins Ctr4-M6 and Ctr5-M6 in the JSY22 strain did not allow these cells to grow on glycerol/ethanol medium. To ensure that the loss of function was not due to mislocalization of Ctr4-M6 and Ctr5-M6, the mutant proteins were tagged with GFP and MYC₁₂ and localized (Fig. 3B). Both mutant proteins were present on the plasma membrane in a similar manner to the wild-type versions of the Ctr4-GFP and Ctr5-MYC₁₂ proteins (Fig. 3B). Taken together, these results demonstrate that, under standard respiratory growth conditions, the presence of at least one N-terminal domain from Ctr4 or Ctr5 suffices for copper assimilation; however, under severe copper-limiting conditions, in the presence of 20 or 25 μ M BCS, the N termini of both proteins are required for copper acquisition to allow respiratory cell growth.

The five Mets motifs of Ctr4 and Ctr4 Met¹²² are functionally redundant

The N terminus of Ctr4 contains five copies of the sequence Met-X₂-Met-X-Met. As shown above, the Ctr4-M6 mutant lacking these sequences was unable to restore respiratory growth when co-expressed with Ctr5-M6. To determine the importance of these motifs in copper acquisition, sequential deletions of this sequence were created in Ctr4 and co-expressed with Ctr5-M6 in strain JSY22. The Ctr4-M1 mutant lacks the first 53 N-terminal residues, while Ctr4-M2 lacks the 93 N-terminal residues, and the mutants harbour five and one Mets motifs, respectively. Co-expression of either *ctr4*-M1 or *ctr4*-M2 with *ctr5*-M6 in a *ctr4* Δ *ctr5* Δ strain allowed the cells to grow on glycerol/ethanol medium, except in the presence of 20 or 25 μ M BCS (Fig. 4). These results suggested that the fifth Mets motif of Ctr4 might be required for respiratory growth. Surprisingly, however, cells expressing the *ctr4*-M5 allele in which the first four Mets motifs had been deleted and the fifth Mets motif substituted with Ala residues exhibited

Fig. 4. The role of the Mets motifs and Met¹²² in Ctr4 function. The strain JSY22 (*ctr4Δ ctr5Δ*) was co-transformed with the pJK148 and pBPade6⁺ vectors alone, *ctr5⁺-myc₁₂wt + ctr4⁺-GFPwt*, *ctr5-M6 + ctr4⁺-GFPwt*, *ctr5-M6 + ctr4-M1*, *ctr5-M6 + ctr4-M2*, *ctr5-M6 + ctr4-M3*, *ctr5-M6 + ctr4-M4*, *ctr5-M6 + ctr4-M5*, or *ctr5-M6 + ctr4-M6*. For simplicity, only the N-terminal regions of the indicated versions of Ctr5-MYC₁₂ and Ctr4-GFP fusion proteins are depicted. The amino acid sequence numbers refer to the position relative to the first amino acid of the indicated protein. Cells were spotted at a density of 3000 cells (5 μl)⁻¹ onto YES media containing glucose or glycerol/ethanol with 0, 5, 10, 20 or 25 μM BCS. FY435, parental wild-type strain.

medium containing 0, 5 and 10 μM BCS (Fig. 4). Moreover, even in the presence of 20 or 25 μM BCS, the cells exhibited growth, although markedly slower than wild-type Ctr4 (Fig. 4). This observation suggests that the five Mets motifs may compensate for the loss of the Met¹²² residue, giving rise to the question of how many Mets motifs are required for such a compensatory effect.

To determine the minimum number and identity of the Ctr4 Mets motifs required for respiratory competence in the absence of Met¹²², a series of N-terminal deletions of Ctr4-M3 was created by removing 58, 69, 81 and 93 amino acid residues to generate Ctr4-M7, Ctr4-M8, Ctr4-M9 and Ctr4-M4, respectively (Fig. 5A). Each of these mutants was co-expressed with Ctr5-M6. Cells harbouring Ctr4-M7 were able to grow on glycerol/ethanol medium, but not at the same level as the wild-type Ctr4 or Ctr4-M3 mutant (Fig. 5A and data not shown). Indeed, the mutant protein (Ctr4-M7) failed to complement the respiratory deficiency in the presence of the copper chelator BCS. The insufficiency of Ctr4-M7 to allow growth in the presence of BCS was not due to mislocalization, since the protein was properly localized at the plasma membrane (Fig. 5B). Cells expressing the

Ctr4-M8 and Ctr4-M9 mutants barely grew on non-fermentable carbon sources (Fig. 5A). This was probably due to mislocalization, since these mutant proteins accumulated in a perinuclear compartment, with very little protein detected on the plasma membrane (Fig. 5B). When the Ctr4-M4 mutant was co-expressed with Ctr5-M6, cells were able to grow on respiratory carbon sources at a level comparable to that observed for Ctr4-M7 (Fig. 5A). Taken together, these data reveal that when all of the five Mets motifs of Ctr4 are present, they can compensate for the Ctr4 Met¹²² mutation, suggesting that these domains perform redundant copper-transport functions in Ctr4.

Increased copper-transport deficiency results from alteration of the Cys-X-Met-X-Met sequence of Ctr5 compared with alterations in the overlapping Mets motifs

To determine the role of the two partially overlapping Mets motifs and the Cys-X-Met-X-Met sequence in Ctr5, we co-expressed the *ctr4-M6* mutant allele in combination with *ctr5* mutant alleles in which the Met and Cys residues of the Met-X-Met-X₂-Met-X₂-Met-X-Met and Cys-X-Met-X-Met

(A)

motifs were mutated to Ala, individually or in combination, by site-directed mutagenesis (Fig. 6). Expression of either M1 or M2 mutants in cells expressing *ctr4*-M6 did not affect cell growth on glycerol/ethanol medium compared to cells co-expressing *ctr4*-M6 and *ctr5*⁺-*myc*₁₂wt (Fig. 6). In contrast, when all Met residues in the two partially overlapping Mets motifs were altered to Ala (Ctr5-M3), the ability of the cells to grow on glycerol/ethanol was compromised in the presence of 10 μM BCS (Fig. 6). To examine a possible role for the Cys-X-Met-X-Met sequence in copper acquisition, we replaced Cys²⁷, Met²⁹ and Met³¹ with Ala (Ctr5-M5). *ctr4*Δ *ctr5*Δ cells co-expressing *ctr5*-M5 and *ctr4*-M6 were unable to grow on glycerol/ethanol medium containing 10 μM BCS, and exhibited only a very little growth in the presence of 5 μM BCS (Fig. 6). This suggests a greater contribution to copper transport by the Cys-X-Met-X-Met sequence compared to the two partially overlapping Mets sequences at the N terminus. The last Met in the Cys-X-Met-X-Met sequence, Met³¹, is positionally conserved with respect to the first transmembrane domain among the known Ctr copper-transport proteins (Puig *et al.*, 2002b), suggesting the possibility that this residue may play an important function in Ctr5-mediated copper acquisition. To assess the importance of the conserved Ctr5 Met³¹, we converted Met³¹ to Ala in the full-length Ctr5wt protein (Fig. 6, Ctr5-M4 mutant). Cells co-expressing this mutant with Ctr4-M6, however, had no defect in their ability to grow on respiratory carbon sources (Fig. 6). Importantly, all of the Ctr5 mutant proteins tagged with MYC₁₂ at the C terminus were localized to the plasma membrane (see Supplementary Fig. S2), and immunoblotting analyses showed that all mutants were expressed at similar levels

(data not shown). Together, these data indicate that the two overlapping Mets motifs and the Cys-X-Met-X-Met sequence of Ctr5 both function in copper acquisition, with a greater contribution from the Cys-X-Met-X-Met sequence during growth under copper-limiting conditions.

Minimal C-terminal regions of the Ctr4 and Ctr5 copper-transport proteins required for copper acquisition

As shown previously (Fig. 3A), when the full-length Ctr5 was co-expressed with Ctr4-M6 in which the first four Mets motifs were deleted and the fifth Mets motif and Ctr4 Met¹²² mutated to Ala, cells were able to grow on glycerol/ethanol, but not in the presence of ≥20 μM BCS. Based on this observation, we tested the effect of further deletions on the N-terminal region of Ctr4 to determine the minimal N-terminal portion of Ctr4 required for Ctr5 function. Removal of the Ctr4 N-terminal region, starting at the initiator codon down to amino acid residue 105, had a similar effect to that observed with the Ctr4-M6 mutant (Fig. 7A). However, when further deletions up to amino acid residues 135 or 166 were created, cells co-expressing these mutants with Ctr5-MYC₁₂ failed to grow on glycerol/ethanol medium (Fig. 7A). Analyses of the localization of the wild-type and truncated versions of the Ctr4-GFP fusion protein by fluorescence microscopy revealed that the ¹⁰⁶Ctr4²⁸⁹-GFP mutant was expressed and localized at the plasma membrane (Fig. 7B). However, the non-functional truncated ¹³⁶Ctr4²⁸⁹-GFP and ¹⁶⁷Ctr4²⁸⁹-GFP fusion proteins were mainly trapped in a perinuclear location and other portions of the secretory compartment, with only a

Fig. 5. Functional dissection of the five Mets motifs in Ctr4. (A) JSY22 (*ctr4Δ ctr5Δ*) was co-transformed with pJK148 and pBPade6⁺ vectors alone, *ctr5⁺-myc₁₂wt + ctr4⁺-GFPwt*, *ctr5-M6 + ctr4-M3*, *ctr5-M6 + ctr4-M7*, *ctr5-M6 + ctr4-M8*, *ctr5-M6 + ctr4-M9*, *ctr5-M6 + ctr4-M4*, or *ctr5-M6 + ctr4-M6*. Growth was tested on fermentable (glucose) and non-fermentable (glycerol/ethanol) agar media and incubated at 30 °C for 5 and 9 days, respectively. Schematic representations (on the left) exhibit only the N termini of the Ctr4 and Ctr5 fusion proteins or their mutant derivatives. FY435, isogenic wild-type strain. (B) *ctr4Δ ctr5Δ* cells co-expressing the indicated alleles were grown to OD₆₀₀ ~1.0. At this OD, BCS (100 μM) was added and the treated cultures were incubated for 3 h at 30 °C, and then visualized for GFP by fluorescence microscopy. The cells were also examined by Nomarski microscopy for cell morphology.

small amount of fluorescence localized at the plasma membrane (Fig. 7B). These results indicate that the presence of the last 184 amino acids (residues 106–289) of the Ctr4 protein is essential for copper acquisition when co-expressed with the wild-type Ctr5-MYC₁₂ protein.

To determine the minimal region of Ctr5 required for copper acquisition with Ctr4-GFP, truncations were created from the N-terminal end of Ctr5 (Fig. 7A). The first mutant,

lacking the first 43 amino acids of Ctr5, allowed growth on glycerol/ethanol media containing 0, 5 and 10 μM BCS when co-expressed with the full-length Ctr4-GFP protein. This truncated version of Ctr5-MYC₁₂ was localized at the plasma membrane (Fig. 7B). However, the ⁷⁷Ctr5¹⁷³-MYC₁₂ mutant, with further deletion to amino acid residue 77, which deleted the first transmembrane domain, failed to support growth on respiratory carbon sources, possibly due to mislocalization of the mutant protein (Fig. 7A, B).

Fig. 6. Functional features of the two partially overlapping Mets motifs and the Cys-X-Met-X-Met sequence in Ctr5. Cells (JSY22) harbouring a *ctr4Δ ctr5Δ* double deletion were co-transformed with empty integrative vectors, *ctr4⁺-GFP_{wt} + ctr5⁺-myc₁₂wt*, *ctr4-M6 + ctr5⁺-myc₁₂wt*, *ctr4-M6 + ctr5-M1*, *ctr4-M6 + ctr5-M2*, *ctr4-M6 + ctr5-M3*, *ctr4-M6 + ctr5-M4*, *ctr4-M6 + ctr5-M5*, or *ctr4-M6 + ctr5-M6*. The co-transformed cells were spotted on media containing 3% glucose (fermentable) or 3% glycerol/2% ethanol (non-fermentable) as carbon sources. Growth rates of the co-transformed cells were compared with the parental strain (FY435). Cells were grown for 5 (glucose) or 9 (glycerol/ethanol) days at 30 °C and photographed. For simplicity, only the N-terminal regions of the indicated variants of the Ctr4-GFP and Ctr5-MYC₁₂ fusion proteins are shown.

The bulk of ⁷⁷Ctr5¹⁷³-MYC₁₂ mutant protein was found in a perinuclear region (Fig. 7B). Therefore, these results suggest that the region encompassing amino acids 44–173 of Ctr5, which contains the three putative transmembrane domains, constitutes a minimal domain that can form a copper-transporting complex with the full-length Ctr4-GFP at the plasma membrane.

DISCUSSION

In this study, we used a stable expression system utilizing integrative plasmids to return wild-type or mutant alleles of

the *ctr4⁺* and *ctr5⁺* genes in a *ctr4Δ ctr5Δ* mutant strain. Using this single-copy expression system, we determined that simultaneous expression of full-length Ctr4 or Ctr4 106–289 and full-length Ctr5 or Ctr5 44–173 is required for proper localization of both proteins at the cell surface. In the absence of Ctr5, Ctr4 is mislocalized within the secretory pathway. Similarly, we found that in the absence of Ctr4, Ctr5 fails to exit an intracellular compartment likely to be the endoplasmic reticulum. Like most Ctr family members, Ctr4 and Ctr5 are rich in methionine residues within their putative N-terminal extracellular hydrophilic domains. Ctr4 harbours five almost perfectly spaced Mets motifs, whereas

Fig. 7. Minimal C-terminal regions of Ctr4 and Ctr5 required to allow growth by respiration. (A) The top panel shows *ctr4Δ ctr5Δ* cells harbouring the wild-type *ctr5⁺-myc₁₂* allele and transformed with ¹*ctr4⁺-289-GFP*, ¹⁰⁶*ctr4⁺-289-GFP*, ¹³⁶*ctr4⁺-289-GFP* or ¹⁶⁷*ctr4⁺-289-GFP* alleles. The bottom panel shows *ctr4Δ ctr5Δ* cells containing the wild-type *ctr4⁺-GFP* allele and transformed with ¹*ctr5⁺-173-myc₁₂*, ⁴⁴*ctr5⁺-173-myc₁₂* or ⁷⁷*ctr5⁺-289-myc₁₂* alleles. Cells were photographed after incubation at 30 °C for 5 days on yeast extract plus glucose plates and 9 days on yeast extract plus glycerol/ethanol plates. FY435 represents wild-type cells. (B) Cells co-expressing the indicated Ctr4 and Ctr5 fusion proteins were visualized by fluorescence microscopy. The Ctr4-GFP fusions are shown in the top panel with the GFP microscope images on the left and the Nomarski images on the right. The epitope-tagged Ctr5 proteins are shown in the bottom panel with the fluorescence (anti-MYC) microscope images on the left and the Nomarski images on the right.

(A)

(B)

Ctr5 contains two copies of partially overlapping Mets motifs and a Cys-X-Met-X-Met sequence, which lies downstream of the two Mets motifs (Labbé *et al.*, 1999; Zhou & Thiele, 2001). To begin to understand the mechanism by which these two proteins deliver copper into the cell, we performed experiments to delineate the respective roles of the Ctr4 and Ctr5 Met-rich sequences in copper assimilation. The analysis of yeast strains expressing Ctr4 and Ctr5 proteins in which the N-terminal regions have been mutated demonstrates that at least one N-terminal region provided by either protein suffices for growth on glycerol/ethanol media containing 0, 5 or 10 μM BCS. However, addition of higher concentrations of BCS (20 and 25 μM) results in an inability to grow on respiratory carbon sources, unless *ctr4* Δ *ctr5* Δ cells co-express both wild-type *ctr4*⁺ and wild-type *ctr5*⁺ genes. Accordingly, these results reveal that although the N-terminal regions of Ctr4 and Ctr5 can function independently in copper transport, the presence of both N termini is required for the most efficient copper-transport activity, especially under severe copper-limiting conditions. Therefore, yeast cells that express both full-length Ctr4 and full-length Ctr5 protein have a distinct growth advantage, when copper is limiting, over cells expressing only one N-terminal region from these copper transporters.

Recent studies have revealed that a single Met or a Met-X-Met motif located approximately 20 amino acids upstream of transmembrane domain one in the Ctr proteins is essential for copper transport (Puig *et al.*, 2002b; Rees *et al.*, 2004b). Ctr4 has one methionine residue (Met¹²²) 22 amino acids from its first transmembrane domain. We assessed the potential role of this residue on Ctr4 function by mutating Met¹²² to Ala. In the context of the full-length protein, no growth-defect phenotype was observed when cells were grown on respiratory carbon sources in the presence of 0, 5 or 10 μM BCS. In the absence of the five Mets motifs (Ctr4-M5), however, the Met¹²² was essential for the growth of cells on non-fermentable media in the absence or presence of BCS (5 or 10 μM). These data suggest that the Ctr4 protein has two potential ways for sequestering copper to the plasma membrane prior to its import through the membrane. One mechanism involves the five Mets motifs, while the second requires Met¹²². In the absence of Met¹²², the presence of all five Mets motifs appears to be crucial for Ctr4 function. Removal of Mets motif 1 (Ctr4-M7) drastically reduces the ability of the cells to grow on standard glycerol/ethanol medium, with no growth on medium supplemented with exogenous BCS. Furthermore, deletion of the first two or the first three Mets motifs (Ctr4-M8 or Ctr4-M9) precludes normal localization of Ctr4 at the plasma membrane. While Ctr4 Mets motif 5 (Ctr4-M4) is sufficient to complement respiratory deficiency on standard glycerol/ethanol medium to the same extent as Ctr4-M7, these cells (Ctr4-M4) fail to grow on respiratory carbon sources when extracellular copper is chelated by BCS.

Within its N terminus, Ctr5 contains two copies of partially overlapping Mets motifs and a Cys-X-Met-X-Met sequence.

The last Met residue (Met³¹) of the latter sequence is located 24 amino acids from the first transmembrane domain. Mutation of this Met residue to Ala in the Ctr5-M4 mutant still allowed the transformed cells to grow on glycerol/ethanol medium when co-expressed with Ctr4-M6. This result suggests that the Ctr5 Met³¹ residue is dispensable for the function of Ctr5. Although we demonstrate that the two partially overlapping Mets motifs of Ctr5 and the Ctr5 Cys-X-Met-X-Met sequence are functionally redundant, the growth of cells lacking the Cys-X-Met-X-Met sequence was more severely limited under conditions of copper deprivation compared to those lacking the two partially overlapping Mets motifs. This may be due to the fact that a Cys rather than a Met residue is found at the first position within the Cys-X-Met-X-Met sequence. It is known that the Cys residue with its external SH group coordinates copper with more affinity than a Met residue (Puig *et al.*, 2002b). Furthermore, the fact that the Cys-X-Met-X-Met sequence is closer to the first transmembrane domain may be important. For the mammalian CPx-type ATPases ATP7A and ATP7B, the metal-binding domains located closer to the first transmembrane domain appear to play a more important role in copper transport (Payne & Gitlin, 1998; Iida *et al.*, 1998; Strausak *et al.*, 1999; Forbes *et al.*, 1999; Huster & Lutsenko, 2003). Perhaps the putative copper-binding motif closest to the first membrane-embedded region can facilitate the translocation of copper through the membrane channel for delivery into the cell.

It is intriguing that the fission yeast genome encodes two structurally related proteins that are interdependent for co-localization to the cell surface and function in copper uptake. This situation is reminiscent of the *FTR1*-encoded iron permease and the *FET3*-encoded multicopper oxidase in *Sac. cerevisiae* that must be co-expressed for stable assembly and function in high-affinity iron transport at the plasma membrane (Askwith *et al.*, 1994; De Silva *et al.*, 1995; Stearman *et al.*, 1996; Severance *et al.*, 2004). Interestingly, *SPAC1F7.07* and *SPAC1F7.08* genes encoding the Ftr1 and Fet3 homologues from *S. pombe*, designated *fip1*⁺ and *fio1*⁺, respectively, must also be co-expressed to restore high-affinity iron transport to a *Sac. cerevisiae* *fet3* Δ mutant strain (Askwith & Kaplan, 1997). Furthermore, the *fip1*⁺ and *fio1*⁺ genes lie adjacent to one another in the *S. pombe* genome and are transcribed divergently, suggesting coordinate regulation (Askwith & Kaplan, 1997; Labbé *et al.*, 1999). What might be the potential advantages for cells to possess two membrane proteins such as Ctr4 and Ctr5 that form a two-component copper-transporting complex at the cell surface? Clearly, our data demonstrate that the presence of the N termini of both Ctr4 and Ctr5 is required for optimal cell growth under conditions in which copper is required, yet limiting. Because the putative extracellular N-terminal regions of both Ctr4 and Ctr5 have a direct role in copper assimilation, this may prevent a complete loss of function resulting from the inactivation of one extracellular N terminus. While the efficacy of copper transport would be diminished, it would still allow the heteroprotein complex

to mediate copper acquisition and transport. It may also be that the heteromeric Ctr4–Ctr5 complex is involved in the ordered assembly of higher-order complexes at the cell surface. The possibility exists that the heteroprotein complex may play a role in protein–protein interactions with, for example, the $\text{Fe}^{3+}/\text{Cu}^{2+}$ reductases or cytosolic carrier proteins. Recently, it has been suggested that Ctr5 could serve to ensure stable assembly of the high-affinity copper-transport complex, because Ctr4 has residues at positions 236 (Phe) and 237 (Leu) in transmembrane domain three that hinder formation of a stable multimeric complex (Aller *et al.*, 2004). Because Ctr5 was originally identified based on its ability to alleviate a block in Ctr4 sorting to the plasma membrane, it is quite possible that the Ctr5 protein is required for Ctr4 folding, hetero-oligomerization and exit from the secretory pathway. Further characterization of Ctr4 and Ctr5 will be required to elucidate how these two transmembrane proteins assemble as a multisubunit complex to migrate through the secretory pathway to the plasma membrane, where the complex can mediate copper uptake into the cell.

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