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Escherichia coli CopA N-Terminal Cys(X)$_2$Cys Motifs Are Not Required for Copper Resistance or Transport

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Escherichia coli CopA is a Cu(I)-translocating P-type ATPase that is involved in copper export and resistance. It is an orthologue of the human Menkes and Wilson disease-related proteins. Each of those two human copper pumps has six N-terminal Cys(X)$_2$Cys sequences, but their function in transport is unclear. CopA has two N-terminal Cys(X)$_2$Cys sequences, GLSC$_{14}$GHC$_{17}$ and GMSC$_{110}$ASC$_{113}$. The requirement of these cysteine motifs was investigated by mutagenesis of the codons for all four cysteine residues, singly and in combination. Cells of a _copA_ deletion strain expressing genes for the mutant genes were nearly as resistant to copper as the wild type. In addition, everted membrane vesicles from cells expressing the mutant _copA_ genes exhibited ATP-coupled accumulation of copper similar to that of the wild type. The results indicate that neither of two N-terminal Cys(X)$_2$Cys motifs is required for either resistance or transport.
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Key Words: **C(X)$_2$C motifs; P-type ATPase; CopA; copper pump; copper resistance.**

P-type ATPases form a large family of cation-transporting pumps (1). Members of one subfamily transport monovalent and divalent cations of hard Lewis acids such as H^+, Na^+, K^+, Mg^{2+} and Ca^{2+} (2). The substrates of members of a second subfamily (3, 4) include the divalent cations of soft Lewis acids such as Zn(II), Cd(II), and Pb(II) (5) and monovalent soft metal cations such as Ag(I) (6) and Cu(I) (7). Copper pump homologues are widespread, including eukaryotic proteins such as the human Menkes (MNK) (8) and Wilson (WND) (9) disease-related proteins and prokaryotic transporters such as the CopA proteins of *Helicobacter pylori* (10), *Enterococcus hirae* (11), and *Escherichia coli* (12).

The *E. coli* protein is required for copper tolerance; disruption of *copA* has been shown to produce sensitivity to copper salts in *E. coli* (12). Resistance is related to CopA-catalyzed copper extrusion from cells. Everted membrane vesicles, which have an orientation opposite to that of intact cells, accumulate ^{64}Cu(I) in an ATP-dependent manner, reflecting copper efflux out of intact cells.

A characteristic feature of these proteins is the presence of N-terminal Cys(X)$_2$Cys sequences. Since peptides containing these sequences have been shown to be capable of binding copper, zinc and other soft metals *in vitro* (13–15), the Cys(X)$_2$Cys motifs have been termed metal binding sites (MBSs) (3). What is the function of the N-terminal these putative MBSs? There are a number of possibilities that are not mutually exclusive. First, it is possible that they confer metal ion selectivity. However, the *E. coli* CopA and the human MNK and WND are monovalent soft metal pumps, while the closely related ZntA is a divalent soft metal pump—yet all have very similar primary sequences in their MBSs. Thus, this motif is probably not sufficient to impart metal ion specificity. Second, the metal binding domains may serve as metal sensors to regulate the pump (16). Third, the MBSs may serve as the initial binding sites. The ion would be transferred to the translocation domain, which may include the conserved CysProCys sequence in the sixth transmembrane segment. Fourth, the MBD may be the site of interaction with metal ion chaperones (17). In eukaryotes soluble metal ion chaperones are required for insertion of copper into copper proteins such as cytochrome oxidase and superoxide dismutase. The yeast ATX1 chaperone also inserts Cu(I) into CCC2, a homologue of the *E. coli* CopA (18). In *Rhodobacter capsulatus,* disruption of the gene for CcoI, a CopA homologue, prevents expression of cytochrome cbb(3) oxidase (19), suggesting that the CopA homologue is required for insertion of copper during biogenesis of the

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oxidase. However, to date no metal ion chaperones have been identified in *E. coli.* Fifth, in eukaryotes the MBSs may be involved in trafficking to the appropriate membrane (20–23). On the other hand, the N-terminal metal binding domains may have a different function in *E. coli,* which lacks intracellular membranes that would require trafficking signals.

The choice of the *E. coli* system for analysis of the role of specific residues of a copper-translocating P-type ATPase has certain advantages. First, it can be difficult to differentiate between effects on trafficking versus catalysis for the eukaryotic copper pumps. The absence of intracellular membranes in *E. coli* avoids this problem. Second, *in vivo* the function of eukaryotic copper pumps relies on interaction with a metal ion chaperone. No metal ion chaperones have as yet been identified in *E. coli* in spite of extensive genetic and genomic analyses. Third, analysis of the function of mutated copper pumps is frequently accomplished by indirect assays in heterologous systems such as the effect of animal copper pumps on Fet3p activity in a *ccc2*Δ strain of *Saccharomyces cerevisiae* (23, 24). In contrast, CopA activity can be directly measured both *in vivo* and *in vitro.*

In this study each of the four cysteine residues in the $GLSC_{14}GHC_{17}$ and $GMSC_{110}ASC_{113}$ sequences of CopA were altered by site directed mutagenesis to alanine residues, producing four single mutants, six double mutants and a quadruple mutant. Another construct had a deletion of residues 8–150. Cells expressing each of the mutant copA genes were examined for copper resistance. *In vivo,* only the N-terminal deletion mutant became sensitive to copper; all of the cysteine-to-alanine substitutions retained copper resistance. *In vitro* accumulation of $^{64}Cu(I)$ was assayed in everted membrane vesicles from cells expressing the mutant *copA* genes. Consistent with the *in vivo* results, each of the substitution mutants retained ATP-driven copper transport, while the N-terminal deleted protein was unable to catalyze copper uptake into everted membrane vesicles. Thus the $Cys(X)_2Cys$ MBSs are not required for the basic resistance and transport properties of the CopA P-type ATPase.

EXPERIMENTAL PROCEDURES

Growth of cells. Cells were grown in Luria–Bertani (LB) medium (25) at 37°C. Ampicillin (50 μg/ml), kanamycin (50 μg/ml), chloramphenicol (30 μg/ml), isopropyl-β-D-thiogalactopyranoside (0.1 mM), 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (80 μg/ml) and L(+)-arabinose (0.0002%) were added as required. To assay inhibition of growth by metal salts, cells were grown overnight in LB, diluted 1:100 in the same medium with the indicated metal salts, and incubated for 6 h for $CuSO_4$ at 37°C with shaking. Growth was monitored from the absorbance at 600 nm. Each strain reached approximately the same final density in the absence of copper, and so the data were normalized to the value in the absence of metal.

Strain construction and plasmids. Standard molecular and genetic techniques were used for strain and plasmid construction (25). Deletion of the entire *copA* gene from the *E. coli* chromosome was accomplished by the method of Datsenko and Wanner (26) using a forward polymerase chain reaction (PCR) primer 5′-AGTCATGCG-TCGATGCCAAATGCGCCACCCTAAAGCAGCGCGATTGTGTAG-GCTGGAGCT-3′, and reverse PCR primer 5′-CCCCTTGCTGGAA-GGTTTAACCTTTATCACAGCCAGTCACCATGGTCCATATGAA-TATCCTCC-3′. The underlined regions are complementary to sequences upstream or downstream of *copA,* and the remaining sequences are complementary to the chloramphenicol cassette. Replacement of the *copA* gene with the chloramphenicol cassette by homologous recombination in strain BW2113 (26) was confirmed by PCR, producing strain GR230. The *copA*-deleted gene was transferred from strain GR230 to strain LMG194 by transduction with P1 bacteriophage, with selection for chloramphenicol resistance, resulting in strain DC194.

To introduce mutations in the coding sequence for the N-terminal cysteines, the first 708 base pairs of the *copA* gene were cloned into plasmid pGEM-T Easy (Promega) by PCR using forward primer 5′-TTAACCATGGCACAAACTATC-3′ and reverse primer 5′-ATGGCCGCCGGCGAAAACCAT-3′. Site-directed mutagenesis was carried out by the Quick-Change method (Stratagene). A *copA* gene with a deletion of codons 8–150 (base pairs 22–449) was generated by PCR and cloned into plasmid pGEM-T Easy using forward primer 5′-TGTTCCATGGCACAAACTATCGACCTGGTGCAGGCGGTGGA-AAAAGCGGG-3′ and reverse primer 5′-ACTGCGGTAAAAATGG-CCGCCGGCGAAAACCATCACTGCCAGGGTTATC-3′. All mutations were verified by sequencing the entire insert. The inserts were excised by double digestion with *Nco*I and *Nae*I and ligated into *Nco*I–*Nae*I digested plasmid pCopA2, in which *copA* is controlled by the arabinose promoter. In pCopA2 *copA* is in-frame with the sequence for the Myc epitope and six histidine codons.

Preparation of everted membrane vesicles. Cells were grown overnight at 37°C in 5 ml LB, diluted 100-fold into prewarmed medium and allowed to grow to an optical density of 0.7 at 600 nm. Cells were induced with 0.0002% arabinose for 2 h at 30°C. Everted membrane vesicles were prepared as described previously (5), except the cells were lysed at 16,000 psi. The membrane vesicles were suspended in a buffer consisting of 10 mM Tris–HCl, pH 7.0, containing 0.25 M sucrose and 0.2 M KCl and stored at −70°C until use. Protein concentrations were determined using a bicinchoninic acid method (27).

Polyacrylamide gel electrophoresis and immunoblotting. Samples were prepared by incubation in sodium dodecyl sulfate (SDS) sample buffer for 30 min at room temperature and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (28). Immunological blotting with antibody (Clonetech) to a six-histidine tag was performed as described previously (29).

^{64}Cu transport assays. ^{64}Cu was obtained from the Mallinckrodt Institute of Radiology, Washington University Medical Center. Transport assays were performed at room temperature as described previously (12). Unless otherwise noted, the reaction mixture (1 ml) contained 40 mM histidine (pH 6.8), 0.2 M KCl, 0.25 M sucrose, 1 mM dithiothreitol, 0.5 mg of membrane protein, 10 μM $^{64}CuCl_2$ (0.5–10 μCi/ml), and 5 mM Na_2ATP or Na_2ADP. The reaction was initiated by addition of 5 mM $MgCl_2$. At intervals, 0.1 ml samples were withdrawn and filtered through nitrocellulose filters (0.22 μm pore size, Whatman). The filters were presoaked in a buffer consisting of 40 mM histidine, pH 6.8, 0.2 M KCl, 0.25 M sucrose, 10 mM $MgSO_4$ and 20 mM $CuCl_2$. Following filtration, the filters were washed with 5 ml of the same buffer, dried, and the radioactivity quantified in a liquid scintillation counter. The values obtained with the assay mixture without membrane vesicles were subtracted from all time points. To correct for differences in expression of the mutant proteins, the amount of CopA in everted membrane vesicles was normalized to that of the wild type by Western blot analysis. Each set of membranes was analyzed at four different concentrations, and a standard curve was constructed from the values obtained by densitometry.

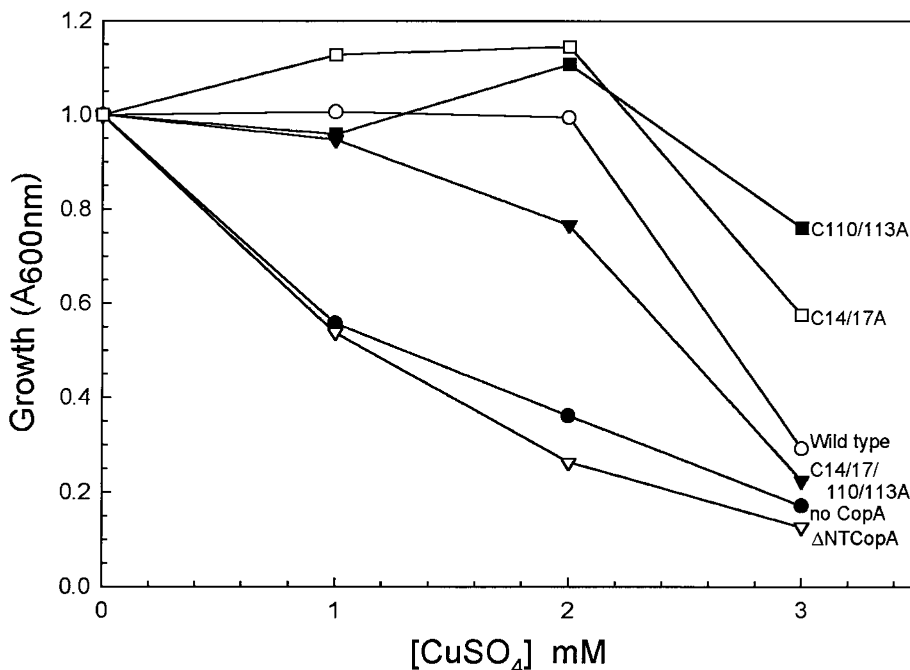

FIG. 1. Copper resistance in *E. coli* expressing wild type and mutant copA genes. Copper ion resistance was assayed in strain DC194 (*copA*) bearing the indicated plasmids, as described under Experimental Procedures. Strains/plasmids: (●), pBADA (vector); (○), pCopA2 (wild type); (□), $pCopA_{C14/17A}$; (■), $pCopA_{C110/113A}$; (▼), $pCopA_{C14/17/110/113A}$; (▽), ΔNCopA.

RESULTS AND DISCUSSION

Effect of substitution of the cysteine residues in the N-terminal Cys(X)₂Cys motifs on resistance to copper. The effect of mutation of the codons for Cys14 and Cys17 in the first $Cys(X)_2Cys$ motif and Cys110 and Cys113 in the second, singly and in combination, on resistance to copper salts was determined. *E. coli* is intrinsically resistant to copper. Deletion of the chromosomal *copA* gene renders cells more sensitive, and complementation by a wild type copA gene on a plasmid increases tolerance. This is especially evident at 1–2 mM (Fig. 1). None of the single cysteine-to-alanine mutations had a significant effect on the ability to grown in the presence of copper sulfate compared to the wild type (data not shown). Indeed, when any three or all four cysteines were mutated, each of the resulting plasmids conferred resistance to up to 4 mM $CuSO_4$ in strain DC194 (Δ*copA*) (Fig. 1). It is not clear whether the differences among the strains are significant. Resistance was specific for copper salts; the mutant plasmids did not confer resistance to Pb(II), Zn(II), Cd(II), Co(II), As(III), or Sb(III) (data not shown). Thus, under the growth conditions employed, none of the four cysteine residues in the two $Cys(X)_2Cys$ motifs in CopA is required either for copper resistance or soft metal ion selectivity. Considering the evolutionary conservation of the motifs, it is possible—perhaps likely—that they have a physiological role in CopA function. In this study resistance was examined only in solid or liquid media under aerobic conditions. A systematic examination of growth conditions might turn up a thus far undetected requirement.

If the $Cys(X)_2Cys$ motifs are not required, is the N-terminal cytosolic domain required at all? The first membrane-spanning region of CopA can be predicted to include residues 179–196. A *copA* gene was constructed in which codons 8–150 were deleted, producing an N-terminally deleted CopA (NCopA). In contrast to the cysteine mutants, this plasmid was unable to confer resistance to copper (Fig. 1). The loss of resistance is not due to lack of expression of the deletion. By Western blot analysis each of the substitutions and NCopA were produced in amounts roughly similar to that of the wild type and were localized in the membrane (Fig. 2). This suggests that the N-terminal cytosolic domain may contribute to CopA function. However, misfolding of NCopA cannot be ruled out. Recently Mitra and Sharma (30) have shown that an N-terminal truncation of ZntA, the *E. coli* Zn(III)/Pb(II)/Cd(II)-translocating P-type ATPase, has nearly wild type resistance to zinc, cadmium and lead, and the soft metal ion selectivity was similarly not affected. While this study clearly demonstrates that the N-terminus of ZntA is not required for ATPase activity, no mutations were introduced into the single $Cys(X)_2Cys$ motif of ZntA, so their role in metal recognition has not addressed. For example, if the cysteines were required for metal-dependent activation of the enzyme, it is

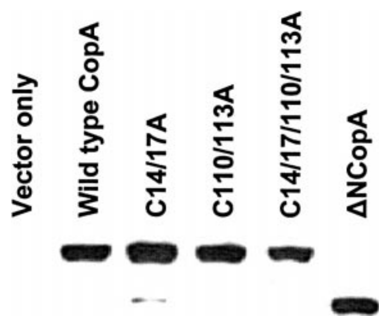

FIG. 2. Expression of wild type and mutant *copA* genes. Cells of strain DC194 (*copA*) with the indicated plasmids were grown and induced, and immunoblotting was performed on membranes as described under Experimental Procedures. Lane 1, pBADA (vector); lane 2, pCopA2 (wild type); lane 3, $pCopA_{C14/17A}$; lane 4, $pCopA_{C110/113A}$; lane 5, $pCopA_{C14/17/110/113A}$; lane 6, ΔNCopA.

possible an N-terminal truncation would be active but cysteine mutants inactive.

Effect of mutations on ATP-dependent ^{64}Cu(I) transport in everted membrane vesicles. While no effect of mutations of the four cysteine residues was observed *in vivo,* more subtle changes might be observed *in vitro.* In a comparison of the ATPase activity of purified ZntA with its N-terminally truncated form, the mutant exhibited a reduced Vmax compared to the wild type (30). Our preparations of purified CopA are not sufficiently active for ATPase analysis. In everted membrane vesicles, however, CopA-catalyzed accumulation of ^{64}Cu(I) can be assayed. Everted membrane vesicles were prepared from cells of strain DC194 (Δ*copA*) expressing wild type and mutant *copA* from genes from a plasmid. Consistent with the *in vivo* results, everted membrane vesicles prepared from NCopA did not take up ^{64}Cu(I). In contrast, everted membrane vesicles prepared from all of the cysteine-substituted mutants exhibited ATP-coupled accumulation of ^{64}Cu(I), and representative data are shown in Fig. 3. In these assays the amount of CopA proteins in membranes was quantified by densitometric analysis of Western blots and normalized to the wild type value. Accumulation in vesicles from both wild type and cysteine-substituted mutants was dependent on the presence of dithiolthreitol and inhibited by ortho-vanadate in a dose-dependent manner (data not shown). The C110/113A mutant reproducibly exhibited both greater resistance and higher levels of uptake than the wild type, but the cause of this apparent increased activity relative to the wild type is not known. Rates of transport were measured at several higher and lower concentrations of ^{64}Cu. Similar uptake was observed in the wild type and mutants at several higher concentrations of copper, suggesting that the V_{max} was not altered by the cysteine substitutions (data not shown). However, because of the short half-life of ^{64}Cu and low rates of uptake, insufficient data were acquired at low copper concentrations to allow for calculation of K_m values.

These results are consistent with those of Voskoboinik and coworkers (31), who examined the effect

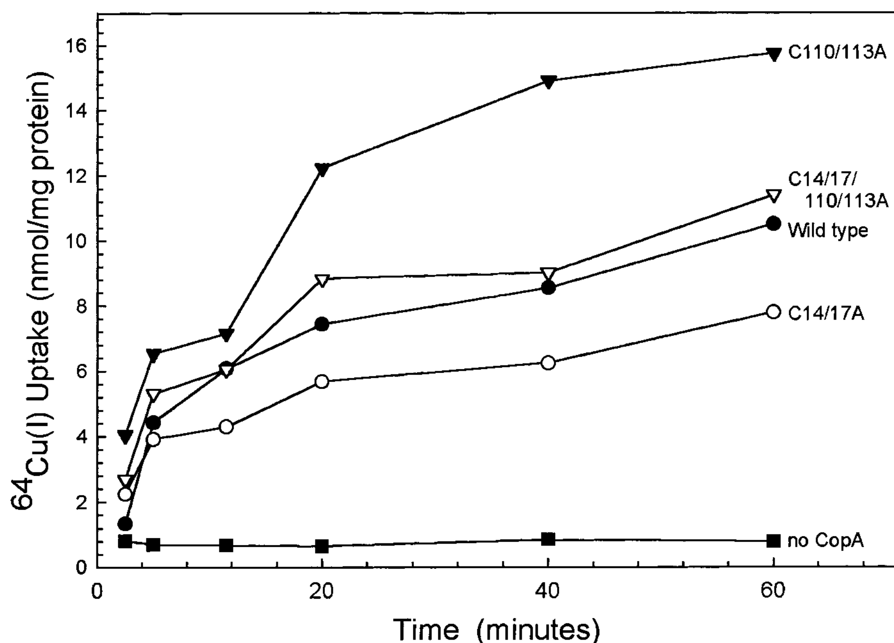

FIG. 3. Uptake of ^{64}Cu in everted membrane vesicles of *E. coli* strain DC194 (*copA*) expressing wild type or mutant *copA* genes. Vesicles were prepared as described under Experimental Procedures. Cells were induced with 0.0002% arabinose as described under Experimental Procedures. Transport was assayed with 10 μM $^{64}CuSO_4$ reduced with 1 mM DTT. Plasmids: (■), pBADA (vector); (●), pCopA2 (wild type); (○), $pCopA_{C14/17A}$; (▼), $pCopA_{C110/113A}$; (▽), $pCopA_{C14/17/110/113A}$.

of cysteine-to-serine mutations in all of the six $Cys(X)_2Cys$ motifs of the human MNK. Wild type and mutant MNK genes were heterologously expressed in CHO cells, and uptake of $^{64}Cu(I)$ was measured in Golgi-enriched membrane vesicles. Membranes from a mutant with cysteine-to-serine substitutions of all six MBSs retained 50–70% of wild type transport activity. However, when the same mutant was heterologously expressed in *S. cerevisiae,* $^{64}Cu(I)$ transport was not observed, even though the MNK protein was still capable of forming a phosphoenzyme intermediate (32). Overall, the results suggest that none of the six MBSs are required for human MNK catalysis, but it is not clear why there is a difference in the activity of the same protein heterologously expressed in two different organisms.

In summary, from the results presented in this paper, it can be concluded that neither N-terminal $Cys(X)_2Cys$ is required for the copper transport properties of CopA, either *in vivo* or *in vitro.* Since a CopA with none of the four N-terminal cysteines catalyzes transport in everted membrane vesicles, it is unlikely that the N-terminus is a metal-responsive regulatory domain. However, while the results do not define a role for the N-terminus, they do validate the use of the *E. coli* system as a model for copper ion-translocating P-type ATPases. Future directions will focus on alternate physiological functions of the N-terminal cysteine motifs and on purification of active CopA to allow examination of the effects of the mutations on ATPase activity.

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