Restriction of Copper Export in *Saccharomyces cerevisiae* to a Late Golgi or Post-Golgi Compartment in the Secretory Pathway*

(Received for publication, May 29, 1997, and in revised form, August 4, 1997)

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The *CCC2* gene in the yeast *Saccharomyces cerevisiae* encodes a P-type ATPase (Ccc2p) required for the export of cytosolic copper to the extracytosolic domain of a copper-dependent oxidase, Fet3p. Ccc2p appears to be both a structural and functional homolog of ATPases impaired in two human disorders of intracellular copper transport, Menkes disease and Wilson disease. In the present work, three approaches were used to determine the locus of Ccc2p-dependent copper export within the secretory pathway. First, like ccc2 mutants, sec mutants blocked in the secretory pathway at steps prior to and including the Golgi complex failed to deliver radioactive copper to Fet3p. Second, also like ccc2 mutants, eps33 and certain other mutants with defects in post-Golgi sorting exhibited phenotypes traceable to deficient copper delivery to Fet3p. These findings were sufficient to explain the respiratory deficiency of these mutants. Third, immunofluorescence microscopy revealed that Ccc2p was distributed among several punctate foci within wild-type cells, consistent with late Golgi or post-Golgi localization. Thus, copper export by Ccc2p appears to be restricted to a late or post-Golgi compartment in the secretory pathway.

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Copper enzymes serve vital functions that involve molecular oxygen (1). In eukaryotic cells, the localization of these enzymes within specific cellular compartments varies with different enzymes and is a crucial determinant of their physiological roles. In both yeast and humans, for example, cytochrome *c* oxidase plays an essential role in oxygen utilization in mitochondria, while Cu,Zn-dependent superoxide dismutase functions in the detoxification of oxygen-derived free radicals in the cytosol. Most of the copper in the human body is in fact found in ceruloplasmin, a multicopper oxidase and glycoprotein secreted from liver cells into the circulation. This copper enzyme appears to function in iron metabolism (2–4). In the yeast *Saccharomyces cerevisiae*, a ceruloplasmin homolog, Fet3p, has been identified that participates in high affinity iron uptake at the cell surface (5). The fact that copper enzymes are localized to various cellular compartments suggests that mechanisms exist for the transport of copper across membranes into these compartments, since it is otherwise difficult to envision how fully folded, copper-loaded proteins might enter membrane-bound compartments from the cytosol. Of particular interest is how copper is transported into the secretory pathway for delivery to secreted or extracellular copper enzymes. In humans, such enzymes carry out a variety of functions, including the biosynthesis of neurotransmitters and the maturation of connective tissue. Specific mechanisms for intracellular copper transport can be expected, given that foodstuffs usually contain only trace amounts of copper, but until recently these mechanisms have been obscure.

In 1993, the genes associated with two inherited disorders of human copper metabolism were identified. One of these disorders, Menkes disease (6–8), is characterized by a deficiency of copper in most tissues. The other, Wilson disease (9–11), is marked by failure of the liver to excrete surplus copper into the biliary tract and to deliver copper to ceruloplasmin. Despite different tissue specificities, both disorders appear to involve a defect in the export of copper from the cytosol (12). The genes associated with both of these disorders encode homologous P-type ATPases, suggesting that these ATPases mediate the export of copper from the cytosol (13). Although functional characterization of these ATPases has proven difficult, some progress has been made in the characterization of a yeast homolog. This homolog, encoded by the *CCC2* gene (14), also appears to export copper from the cytosol, since *ccc2* mutant cells fail to deliver copper to the ceruloplasmin homolog, Fet3p, a membrane-bound glycoprotein whose copper-binding domains lie outside the cytosol (15). Despite a failure to synthesize the holoprotein form of Fet3p, *ccc2* mutant cells still produce wild-type amounts of the apoprotein form of Fet3p, and copper uptake remains unimpaired (15). Thus, *ccc2* mutant cells exhibit signs of defective copper export into the secretory pathway that aptly model those observed in Menkes disease and Wilson disease.

The aim of the present work was to characterize where copper enters the secretory pathway in yeast, using the genetic tools that are especially advantageous in this organism. Using a variety of yeast mutants with known defects in protein trafficking, the copper export activity of Ccc2p was assessed using biochemical and physiological assays of the copper content of Fet3p, and Ccc2p itself was visualized by immunofluorescence microscopy. It might be conjectured that copper export would occur within the endoplasmic reticulum, given the common requirement for metal cofactors in protein folding (16), or within the Golgi complex, in view of evidence that manganese is exported from the cytosol in yeast by a Golgi-resident P-type ATPase, Pmr1p (17). The studies reported here suggest instead that Ccc2p-dependent copper export is restricted to a late- or post-Golgi compartment in the secretory pathway.

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**EXPERIMENTAL PROCEDURES**

**Yeast Strains**

The *ccc2::URA3* and *Δfet3::TRP1* cells were in the parental background YPH252 as described (15). The *Δstr1::TRP1* cells were in the same background but opposite mating type (strain 42C) (18). The
Cloning of Ccc2p—The Ccc2p coding region was amplified by polymerase chain reaction (28) using genomic DNA from strain BY4742 (29) and the oligonucleotides 5'-CTGGTTCGGCCCAACCAAGACTTCTTCGTTTGA-3' and 5'-CTGGTTTCGGCCCAACCAAGACTTCTTCGTTTGA-3'. Purified polymerase chain reaction products were introduced by ligase-independent cloning (30) into a low copy number yeast expression vector based on the CEN- and HIS3-containing vector, pRS413 (Stratagene) (29). This vector (pDY207) was designed to fuse the 3'-ends of cloned coding sequences with sequences encoding three tandem copies of the influenza hemagglutinin (HA) epitope tag (31) followed by two stop codons and the Corynebacterium glutamicum ccc2 coding region together with 395 bp of 5'-untranslated region and 51 bp of 3'-untranslated region. Expression of a Ccc2p-containing plasmid (pDY228) or an empty expression vector (pDY225) in yeast cells was accomplished by growth at 30 °C in the defined medium used for copper autoradiography except with CuSO4 added to 0.1 μM (for ccc2 cells with epitope-tagged Ccc2p) or 5 μM (for vps cells) and with uracil omitted. Immunofluorescence techniques were essentially as described (32), except that the primary antibody (1:2000) was a purified monoclonal antibody directed against the HA epitope tag (HA.11, Babco, Berkeley, CA) (33); the secondary antibody (1:200) was a Cy3 conjugate (Jackson ImmunoResearch, West Grove, PA). Little specific signal was obtained with the anti-HA monoclonal antibody 12CA5 or a Texas Red conjugate.

Results

Fet3p is a Cell Surface Protein That Requires Ccc2p for Copper Loading—The importance of ccc2 in the delivery of copper to Fet3p was demonstrated previously in studies that monitored the oxidase activity of Fet3p in ccc2 mutants (15). In the experiments depicted in Fig. 1A, copper delivery to Fet3p was demonstrated directly by labeling cells in vivo with radioactive copper. Membrane extracts from such cells were analyzed by electrophoresis for radioactivity co-migrating with Fet3p-dependent oxidase activity. A single radioactive species of the expected mobility was detected in extracts from wild-type cells but not in extracts from ccc2 and fet3 cells (Fig. 1A). Previous studies also established that delivery of copper to Fet3p requires at least two other cell surface proteins. The plasma membrane copper transporter, Ctr1p, appears to be required for the biosynthesis of all cellular copper proteins, Fet3p in particular (26), while the plasma membrane iron transporter, Ftr1p, forms a molecular complex with Fet3p, as suggested by the fact that both Ftr1p and Fet3p must be expressed in order for Fet3p to be loaded with copper and for Ftr1p to leave the endoplasmic reticulum (18). As expected, no radiolabeled Fet3p was detected in ctr1 or ftr1 cells.4

The ability of copper added in vitro to reconstitute oxidase activity in membrane extracts from ctr1, ccc2, and ftr1 (but not fet3) cells (15, 18) suggested that Fet3p is present as an apoprotein in these cells. As shown in Fig. 1B, Fet3p polypeptide in ccc2 and ftr1 cells could also be demonstrated directly using

1 A. Dancis, unpublished observation.
2 The abbreviations used are: MES, 4-morpholineethanesulfonic acid; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; HA, hemagglutinin; WT, wild type.
3 D. S. Yuan unpublished observations.
4 D. S. Yuan, manuscript in preparation.
in wild-type and with proteinase K. This treatment destroyed most of Fet3p were digested to form spheroplasts and subsequently incubated terminus of Fet3p. A polyclonal antibody directed against a peptide in the carboxyl a resistance to digestion, greater enzymatic detection of total Fet3p. Fet3p was detected either by an face proteolysis of Fet3p.

To determine whether Fet3p is a cell surface protein, cells were digested to form spheroplasts and subsequently incubated with proteinase K. This treatment destroyed most of the Fet3p in wild-type and ccc2 cells, but Fet3p in ftr1 cells was largely resistant to digestion (Fig. 1B). These findings indicated that in wild-type cells Fet3p is found primarily at the cell surface, in keeping with the role of Fet3p in high affinity iron uptake (5) and with other studies of Fet3p involving proteolytic digestion of spheroplasts (34). The resistance to digestion, greater electrophoretic mobility, and decreased abundance of Fet3p in ftr1 cells relative to Fet3p in wild-type or ccc2 cells (Fig. 1B) were consistent with previously described genetic and physiological evidence, suggesting that Fet3p is retained in the endoplasmic reticulum in ftr1 cells (18). The fact that Fet3p in ftr1 cells also lacks copper therefore suggested that Fet3p is loaded with copper at a stage in the secretory pathway beyond the endoplasmic reticulum.

Delivery of Copper to Fet3p Requires Transit into the Golgi Apparatus—Temperature-sensitive sec mutants have been widely used to study the effects of defined blocks in the secretory pathway (19, 35). Several of these mutants were used here to demarcate the stages in the secretory pathway within which Fet3p is loaded with copper. Cells were brought to nonpermissive temperature to arrest the transit of Fet3p at a defined stage in the secretory pathway. These cells, which had also been starved for copper, were then incubated with radioactive copper to label only the Fet3p holoprotein synthesized at nonpermissive temperature. Using this assay, it was found that Fet3p-associated radioactivity was virtually absent in cells with secretory blocks at stages preceding, or including, intragolgi transport (sec23, sec18, and sec7) or exocytosis (sec1). In contrast, Fet3p-associated radioactivity was readily detected in cells with secretory blocks at stages involving the transport (sec4) or exocytosis (sec1) of secretory vesicles.

The failure in early sec mutants for copper to be delivered to Fet3p was not due to a failure of copper to enter the cells at nonpermissive temperature. 12–30% of added radioactivity was recovered in cellular homogenates from both wild-type and mutant cells (cell walls that might have bound copper (22) were first removed). ctr1 mutant cells, which lack high affinity copper uptake (26), retained only 0.2% of added radioactivity, indicating that copper was being delivered to Fet3p in sec mutant cells primarily by CTR1-dependent mechanisms.

The failure in early sec mutants for copper to be delivered to Fet3p was also not due to a failure to synthesize Fet3p at nonpermissive temperature. Secretory blocks induced in sec mutants generally do not affect protein synthesis for several hours, despite the accumulation of newly synthesized proteins in intracellular vesicles or organelles (19). When protein synthesis was allowed to proceed at nonpermissive temperature, the sec mutants studied did, in fact, accumulate forms of Fet3p...
that were resistant to proteolytic digestion of intact spheroplasts (sec18 and sec21 (Fig. 2B); sec23). Fet3p accumulating in cells blocked in endoplasmic reticulum-to-Golgi transport (sec18, sec21, and sec23 mutants) would be unable to undergo glycosylation within the Golgi complex, and indeed Fet3p from these cells exhibited greater electrophoretic mobility than Fet3p from wild-type cells (Fig. 2B). Although protease-inaccessible, high mobility Fet3p was also observed in sec18 and sec23 cells treated with cycloheximide before being heated to nonpermissive temperature (Fig. 2B), these findings do not contradict the assertion that Fet3p biosynthesis continues at the nonpermissive temperature in these cells; most likely, they simply indicate that the sec18 and sec23 strains used in these experiments retain a residual secretory block even at the permissive temperature.

**Delivery of Copper to Fet3p Requires Many Genes Involved in Vacular Protein Sorting**—The finding that Fet3p is loaded with copper in a late Golgi or post-Golgi compartment led to an examination of mechanisms of vacuolar protein sorting for their possible relevance to the processes that deliver copper to Fet3p, since vacuolar protein sorting is one of the principal functions of these compartments (36, 37). It was known that certain vacuolar protein sorting (vps) mutants with so-called class C or class D morphologic features (exhibiting multivesicular vacuolar morphology and defective vacular inheritance, respectively) (20, 35) have a defect in respiratory function, manifest as the inability to use glycerol or ethanol as a metabolic substrate, e.g. vps18 (pep3) (38), vps6 (39), and vps11 (end1) (40). A connection between the respiration-deficient phenotype and the biochemistry of Fet3p and Ccc2p in these cells was revealed by the observation that ethanol-dependent growth of a vps33 (class C) mutant (41) could be significantly enhanced by supplementing the growth medium with either copper or iron (Fig. 3A), a phenotype also observed in ccc2 cells (15). The ability of copper to restore respiratory function was FET3-dependent (Fig. 3A). These effects of copper on respiratory competence were closely reflected in the ability of copper to restore a defect in high affinity iron uptake (Fig. 3B) and to restore Fet3p-dependent oxidase activity (Fig. 3C) in the same cells. Yet cellular copper uptake, as measured with radioactive copper, and cytosolic Cu,Zn-superoxide dismutase activity in vps33 cells were similar to the activities in wild-type cells, and Fet3p polypeptide could also be demonstrated in these cells by its oxidase activity after reconstitution with copper in vitro (Fig. 3C). (The decreased electrophoretic mobility of Fet3p in the copper-treated extracts in Fig. 3C may be attributable to artifactual copper-induced cross-linking of Fet3p during storage, since no mobility shift was observed when copper was withheld from cell homogenates and membrane extracts until just before electrophoresis.) Thus, vps33 cells exhibited several phenotypes closely resembling those of ccc2 cells, each traceable to a specific defect in Ccc2p-dependent copper export (15).

A survey of vps mutants for the phenotype of copper-correctable iron deficiency revealed many other vps mutants with this phenotype. The class C and class D mutants were most severely affected, but several other mutants of other classes were also impaired to various degrees (Fig. 4A). It should be noted that the phenotypes of these mutants are not necessarily those of deletion mutants (e.g. compare Ref. 20 and 42 with respect to carboxypeptidase Y secretion in cells with spontaneous and null alleles of VPS16).

To examine the biochemical significance of the copper-correctable iron uptake phenotype, ccc2 cells were compared with ccc2 ctr1 cells for their ability to regain iron uptake under conditions of limited copper supplementation. Deletion of the CTR1 gene had no discernible effect on the detailed concentration dependence of the copper correction phenotype (Fig. 4B). Because the CTR1 gene product is responsible for almost all copper uptake under low copper conditions (radiocopper experiments; see above and Ref. 26), copper uptake across the plasma membrane is unlikely to be a determinant of the copper correction phenotype in these cells. Thus, at least in ccc2 cells, the extent to which iron uptake defects are correctable by copper can probably be regarded as a measure of the degree to which Fet3p exists as an apoprotein at the cell surface.
The close resemblance of copper-dependent phenotypes in \textit{vps} mutant cells to those found in \textit{ccc2} cells suggested that Ccc2p resides in, and presumably functions within, a cellular compartment affected by mutations in the vacuolar protein sorting machinery. To visualize this compartment directly, an epitope (HA)-tagged derivative of Ccc2p was expressed in representative \textit{ccc2} and \textit{vps} mutant cells, each exhibiting severe iron uptake defects (0–5\% of wild type). This modified Ccc2p, expressed under its own promoter in a low copy number vector, was capable of restoring high affinity iron uptake and growth in iron-chelating medium in a \textit{ccc2} deletion mutant.\textsuperscript{3} In such cells, the HA-tagged Ccc2p was visualized by indirect immunofluorescence microscopy with antibody directed against the HA epitope and found to be distributed among several punctate foci (Fig. 5A). This pattern of distribution was clearly not perinuclear and instead resembled that of the late-Golgi protein, Kex2p (23). In wild-type cells grown with added copper, the HA-tagged Ccc2p appeared concentrated adjacent to vacuolar membranes (Fig. 5E), while in congenic \textit{vps1}, \textit{vps15}, and \textit{vps33} cells also grown in added copper (to restore their iron and copper status to that of wild-type cells), the HA-tagged Ccc2p was distributed among smaller and more numerous granules.
Intracellular Compartmentalization of Copper Export in Yeast

The different patterns of distribution observed for the HA-tagged Ccc2p expressed in ccc2 (Fig. 5A) or wild-type (Fig. 5E) cells were not attributable to the CCC2 copy number (1 versus 2) or to the copper concentration in the growth medium (0.1 versus 5 μM), since no changes were seen when HA-tagged Ccc2p was expressed in wild-type cells congenic to the ccc2 strain used or when the copper concentration was altered.3

DISCUSSION

In the present work, yeast mutants with genetically determined defects in protein trafficking were used to localize Ccc2p-dependent copper export. Temperature-sensitive sec mutants were used to show that copper export requires integrity of the early secretory pathway. In particular, copper export required function of the intra-Golgi protein encoded by the SEC7 gene, while the SEC4 and SEC1 gene products involved in transport and fusion of secretory vesicles were dispensable. These findings served to localize copper export to a late Golgi or post-Golgi compartment and led to the finding that Ccc2p is aberrantly distributed in a subset of vps mutants with defects in post-Golgi sorting, thereby suggesting that Ccc2p functions in a post-Golgi compartment diverted from the secretory pathway. A model encompassing these and previous (18) findings is presented in Fig. 6, in which Fet3p reaches this post-Golgi compartment as a complex with Pfr1p (18), receives copper from Ccc2p, and then completes its transit to the plasma membrane by mechanisms involving the SEC4 and SEC1 gene products (Fig. 2B).

It remains to be established whether Ccc2p is in fact localized to a sorted post-Golgi compartment or whether it is actually localized to an unsorted late-Golgi compartment within the constitutive secretory pathway. The effects of vps mutations on copper export are most simply interpreted as showing that Ccc2p functions in a sorted post-Golgi compartment, since by definition vacuolar protein sorting involves the sorting of vacuolar proteins from the constitutive secretory pathway. In this view, Fet3p would travel to this compartment for copper loading and then proceed to the plasma membrane. This maturation pathway for Fet3p apparently occurs independently of any protein sorting motifs that may exist in the membrane-spanning or cytosolic domains of Fet3p, since truncation of those domains does not affect copper loading and secretion.3 Although novel in yeast, such pathways are well described in mammalian cells in connection with major histocompatibility complex class II antigen presentation (e.g. Refs. 43 and 44) and with the biogenesis of synaptic vesicles (e.g. Ref. 45). Interestingly, two copper enzymes involved in mammalian neurotrans-
Intracellular Compartimentalization of Copper Export in Yeast

25793

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doi: 10.1074/jbc.272.41.25787

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