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CELLULAR COPPER TRANSPORT

Christopher D. Vulpe and Seymour Packman***
Department of Biochemistry and Biophysics* and Department of Pediatrics,**
University of California, San Francisco, California 94143-0748

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CONTENTS

ABSTRACT

Cellular copper transport processes are required by all organisms for correct utilization in cell biochemical processes and avoidance of the toxicity of copper excess. Copper import into bacterial, yeast, and mammalian cells requires the coordinate function of proteins with both metal-binding and catalytic domains in mediated transport steps. Following entry, detoxification mechanisms found across species include the binding of copper to specific proteins (e.g. metal-

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lothioneins) and the transfer of copper into isolated cell compartments (e.g. periplasmic space, lysosome). Multiple proteins mediate intracellular transfers in bacteria, and glutathione may play a major role in cytosolic copper delivery to cuproenzymes in mammalian cells. Study of two human disorders of copper transport, Menkes disease and Wilson disease, led to the identification of an important category of proteins mediating cell copper export. The Menkes and Wilson disease gene products are copper-transporting ATPases of the P type, with ATPase domains and N-terminal metal-binding amino acid motifs that are evolutionarily conserved in unicellular and mammalian organisms. These observations suggest that yeast and bacterial copper transport proteins, or individual domains of these proteins, may generally have homologues in mammalian systems.

INTRODUCTION

All living organisms, from bacteria to humans, require dietary copper (element 29) for continued growth and development. This nutritional requirement stems from the essential role of copper in the function of the numerous cuproproteins. However, copper in excess of cellular needs mediates free radical production and direct oxidation of cellular components, resulting in detrimental effects. A critical balance must therefore be maintained by specialized cellular transport mechanisms that regulate intracellular copper content.

Copper transport in mammalian cells can be divided into three discernible but interrelated steps: copper uptake, intracellular copper distribution and utilization, and copper export. To understand each process, one must define the phenomenon, measure the step in isolation, and identify the components involved. Although proteins in the transport and utilization of copper have in some instances been identified, our current knowledge of the cellular processes is limited.

Two well-defined copper uptake pathways are used by mammalian cells. Copper bound to ceruloplasmin, and copper not bound to ceruloplasmin, enter the cell via initially distinct but ultimately convergent paths. In contrast to the uptake of other cations, such as transferrin-bound iron, the uptake of copper into the cell is not accompanied by any ligand. The protein components responsible for these entry steps, save for putative ceruloplasmin receptors, remain unidentified.

The processes controlling the distribution of copper to different cellular compartments and its delivery to copper proteins are poorly understood. Metallothionein (MT) may have a more limited role than originally suspected, and other intracellular copper-binding proteins or glutathione (GSH) may instead function in copper distribution. A closer examination of inherited disorders that possibly involve defects in intracellular copper transport, such as Indian

childhood cirrhosis (ICC) in humans or toxic milk in mice, should ultimately improve our understanding of the mechanisms involved.

The human inherited disorders, Menkes disease and Wilson disease, spurred interest in and contributed significantly to our understanding of copper export. Copper accumulation in the liver of patients with Wilson disease and in cultured cells of every other cell type in Menkes disease results from defective copper export. We have only recently learned that the two diseases result from defects in homologous genes with similar function(s) expressed in different tissues. Both genes code for copper-transporting P-type ATPases, a class of integral membrane proteins that transport cations across cellular membranes in organisms as diverse as bacteria and humans. The mottled mouse and the Long-Evans Cinnamon (LEC) rat, long thought to be animal models of Menkes disease and Wilson disease, respectively, were confirmed as animal homologues by molecular cloning studies. Insight into the mechanism of copper export from cells will come from the ongoing characterization of these proteins.

Copper transport is required by all organisms. Study of copper transport in bacteria and yeast has identified proteins involved in aspects of copper transport, such as copper uptake, for which no mammalian proteins have been identified. These unicellular systems therefore provide a broad perspective on copper transport, suggesting experimental approaches in mammalian cells and identifying proteins with potential mammalian homologues. In this review, we first consider selected aspects of copper transport by bacteria and yeast. Mammalian cell copper uptake, intracellular copper distribution, and copper export are then discussed individually. Mammalian metal-responsive transcriptional regulation is not discussed in this review; the reader is referred to reviews on this topic (129, 195). The reader is also referred to other reviews for details of organismal copper transport (62, 98). We feel that a challenge in this field is to proceed from the identification and delineation of transport steps toward the actual identification of the proteins and mechanisms responsible for the uptake, distribution, utilization, and export of trace metals by mammalian cells.

COPPER TRANSPORT IN UNICELLULAR ORGANISMS

An understanding of copper metabolism in unicellular organisms will help elucidate mammalian copper metabolism. For bacteria, yeast, and mammalian cells, copper is a vital yet deadly element. The complex multicomponent copper handling systems identified in bacteria and yeast suggest the existence of a similarly complex system in the mammalian cell. Functional units in bacteria and yeast are likely to have functional, if not structural, homologues in mammals. In this review, we discuss representative systems (see Figures 1 and 2 and the section on P-type ATPases).

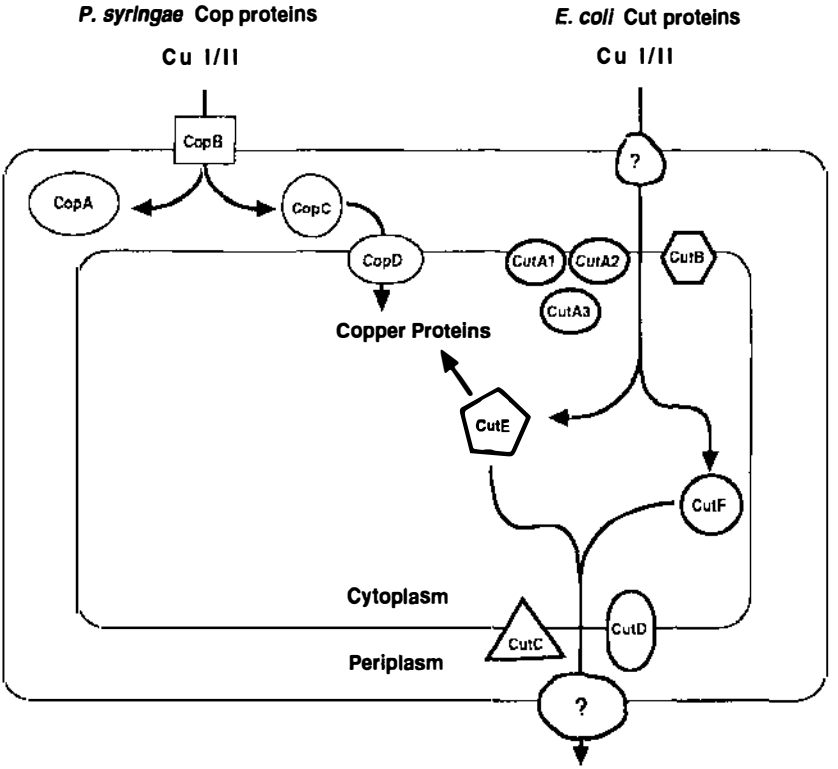

Figure 1 Examples from bacterial copper transport. CopB of *Pseudomonas syringae* transports copper into the periplasmic space, where it is bound by CopA and CopC. CopA binds copper in the periplasm and may have both a storage and an enzymatic function. CopC and CopD cooperate in the transport of copper to the cytosol. In *Escherichia coli,* an additional system, the Cut proteins, are depicted. CutA1–3 and CutB cooperate in transport process across the inner membrane. CutE and CutF deliver copper to cuproproteins and to the export system, CutC and CutD. (Adapted with permission from References 13 and 26.)

Bacterial Transport: Pseudomonas syringae

Resistance of *P. syringae var. tomato* to copper salts sprayed on crops for pest management led to the identification of the *cop* operon (20). The blue color of *P. syringae* colonies grown on copper reflects copper sequestration in the periplasm by proteins encoded by the *cop* operon, *copABCDRS* (25). In one model based on mutational analysis and direct biochemical studies and localization (Figure 1), CopB transports copper across the outer cell membrane into the periplasm. Periplasmic CopA binds 11 copper atoms to multiple copper binding sites. CopA and CopB contain several novel eight–amino acid motifs, X-His-X-X-Met-X-X-Met, which may bind copper (20). A multicopper oxi-

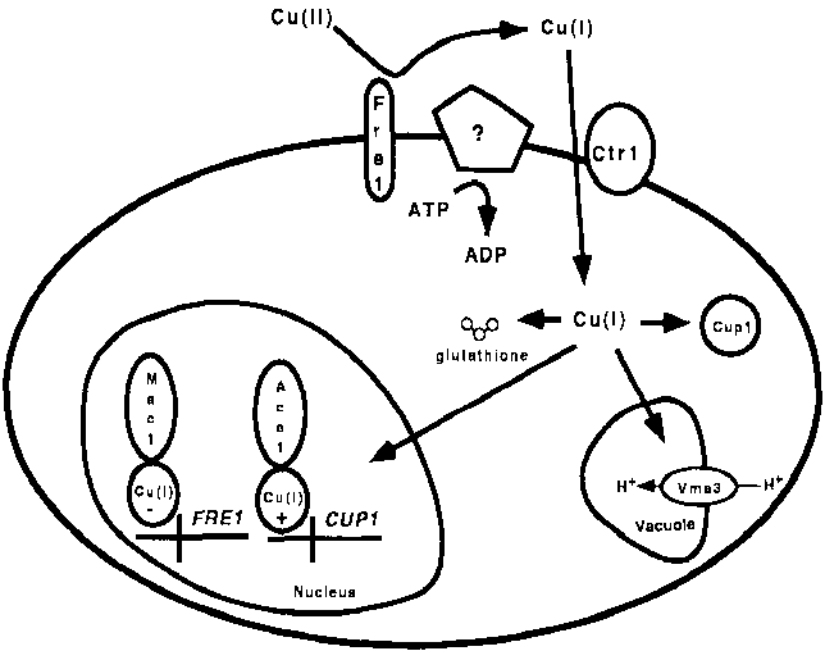

Figure 2 Model of *Saccharomyces cerevisiae* copper transport. An extracellular-facing reductase, Fre1, converts Cu(II) to Cu(I). Cu(I) is transported into the cell in an energy-dependent process involving at least Ctr1. Intracellular copper is bound to GSH and Cup1 (yeast copper MT). Ace1 and Mac1 are copper regulatory proteins that modulate transcription of *CUP1* and *FRE1*, respectively, in response to intracellular copper. Entry of copper into vacuoles is dependent on the vacuolar H^+-ATPase, of which Vma3 is a subunit.

dase signature domain (such as that in ceruloplasmin) is also present in CopA, although biochemical confirmation of an enzymatic function is lacking (26). Periplasmic CopC and inner cell-membrane CopD cooperate in copper uptake into the cell from the periplasm (26).

Bacterial Transport: Escherichia coli

Both plasmid and chromosomal genes govern copper homeostasis in *E. coli*. Six chromosomal genes or complementation groups, *cutA–F*, are likely involved in cellular distribution (144). The plasmid-encoded system, *pcoABCDRS*, may constitute a copper-resistance system utilized in conditions of copper stress (145). The predicted Pco proteins are structurally similar to the predicted Cop proteins from *P. syringae* (13), but the copper resistance mechanisms differ. In high copper, *P. syringae* accumulates copper in the periplasm. In contrast, *E. coli* containing the *pco* operon plasmid actually

accumulates less copper than nonresistant *E. coli* (144); the *pco* genes either prevent the entry of copper or increase copper export.

Genetic analyses of conditional copper-sensitive *E. coli* mutants and their copper import and export phenotypes suggested a model for the role of each protein (Figure 1) (13, 144). CutA and CutB may be involved in copper import: The *cutA* mutant increases the maximum rate of copper import, whereas the *cutB* mutant requires extra copper for growth and accumulates less copper than does wild-type *E. coli*. A three-gene operon encoding two inner-membrane proteins and a cytoplasmic protein of unknown function complements the *cutA* mutant (105). *CutC* and *cutD* mutants show increased copper accumulation and may be involved in energy-dependent copper export. CutE and CutF may deliver copper to copper proteins and to the export system. The recently cloned *cutE* gene predicts a 512–amino acid cytoplasmic protein with a single X-His-X-X-Met-X-X-Met motif similar to the motifs in CopA and CopB from *P. syringae* (143). The recent characterization of protein components such as CutA1–3 and CutE identified novel proteins involved coincidentally in areas of copper transport for which there are as yet no known mammalian counterparts (see below).

Yeast Copper Transport

Copper uptake in *Saccharomyces cerevisiae* appears to be a carrier-mediated energy-dependent process (97). Transport is independent of yeast copper MT, Cup1, and is relatively selective for copper (zinc or nickel only partially inhibit copper uptake). Copper may enter the cell as ionic Cu(I) and then transits to intracellular proteins or compartments (96, 97).

Unexpected insight into copper transport came from the study of iron transport in *S. cerevisiae* (22). Three recently identified genes, *CTR1, FRE1,* and *MAC1,* may play dual roles in copper and iron homeostasis in *S. cerevisiae. CTR1* was identified in a screen for mutants with decreased iron uptake (31). Surprisingly, increased exogenous copper restored iron transport of *ctr1* mutants. Ctr1 defects resulted in primary decreased copper uptake, with a secondary effect in iron transport that was probably due to reduced activity of Fet3, a multicopper oxidase required for iron uptake (6). The *CTR1* gene has no significant overall homology to any known gene. Ctr1 is a plasma-membrane protein with possible metal-binding motifs, including 3 repeats of a 19–amino acid motif containing 4 methionines each, and 11 double methionine motifs, some of which are contained within the extended motif. The second motif, Met-X-X-Met, is similar to the proposed bacterial copper-binding motifs in the (periplasmic) CopA and CopB proteins from *P. syringae* and in the PcoA and CutE proteins of *E. coli*. Thus, the functional and sequence data suggest that Ctr1 is a novel copper-transport protein.

Two additional proteins involved in iron uptake may also play a role in the

uptake of copper. Fre1, an extracellular-facing iron reductase required for iron uptake (30), is also a copper reductase and is probably involved in copper uptake (74). Transcription of the *FRE1* gene is negatively regulated by increased levels of both copper and iron (30, 74), and copper ions also decrease the iron reductase activity of Fre1, suggesting a direct competition for Fre1 by the cations (92). A newly identified regulatory protein, Mac1 (74), may modulate this transcriptional control of *FRE1* in response to copper. Mac1 may therefore regulate copper uptake via regulation of the expression of the copper reductase *FRE1*.

The study of the intracellular fate of copper in *S. cerevisiae* has focused on MT. The data on copper homeostasis from study of yeast copper MT, Cup1, and its copper-specific induction by the Ace1 protein have been summarized (194). The *CUP1* locus is not essential for yeast viability but is necessary for growth in the presence of elevated copper (60). This finding suggests a detoxification function for the Cup1 protein rather than a role in copper delivery to copper proteins. The cellular components responsible for the intracellular routing of copper are unknown, but these functions may be accomplished by GSH and phytochelatins (82). The demonstrated copper-, cadmium-, and zinc-induced synthesis of phytochelatins in *S. cerevisiae* is consistent with a role in copper homeostasis, although the function of phytochelatins, especially when MT is present, remains to be defined (192).

Fortuitous insight into organelle copper transport and possibly copper export came from the study of *S. cerevisiae* mutants with defective vacuoles, the yeast equivalent of the lysosome (42). Yeast with defects in the *VMA3* gene, which encodes a subunit of the vacuolar H^+-ATPase, are hypersensitive to copper, suggesting that the vacuole normally is involved in copper detoxification or export. This observation points to the need to address the effect of other vacuolar mutants on copper sensitivity.

Conclusions

Copper transport in bacteria and *S. cerevisiae* highlights the complexity of copper metabolism and suggests avenues for future work in mammalian systems. Several novel potential copper-binding motifs are shared by copper-binding proteins. A methionine-rich motif, X-His-X-X-Met-X-X-Met, was identified in the *P. syringae* CopA and CopB proteins, the *E. coli* PcoA and CutE proteins, and the *S. cerevisiae* Ctr1 protein. The conservation of this motif makes it likely that mammalian cells also take advantage of its copper-binding properties. A key feature of bacterial and *S. cerevisiae* copper transport is the cooperation of several proteins. The CutA and CutB proteins both play a role in *E. coli* copper import, whereas the pairs of proteins CopC and CopD or CutC and CutD cooperate in bacterial copper export. Both Ctr1 and Fre1 are required for copper uptake in *S. cerevisiae,* with Fre1 performing the copper

reduction necessary for uptake. Bacteria, *S. cerevisiae,* and mammalian cells all utilize the sequestration of copper to handle excessive copper: *P. syringae* retains copper in the periplasm, and eukaryotic MTs bind excess copper. The *vma3* mutant points to a role for vacuoles or lysosomes in copper metabolism, possibly in storage or detoxification. Structural and functional homologies between unicellular organisms and mammals are frequently found, as described below for the Menkes and Wilson disease genes (*MNK* and *WND,* respectively). Examining bacteria and yeast for clues therefore may help explain mammalian copper transport.

MAMMALIAN COPPER TRANSPORT: CELLULAR UPTAKE

The mammalian cell utilizes at least two pathways for copper uptake. In the first, the cell imports copper from the plasma copper protein, ceruloplasmin. Copper bound to albumin or histidine, or unbound copper, enters by a second route, herein designated the "free" copper pathway. Both processes are mediated by energy-independent facilitated transport, result in the entry of copper but not the extracellular ligand, and contribute copper to cuproproteins within the cell (18, 102). The identification of the proteins involved and the elucidation of the relationship between the two processes are challenges for future research.

Ceruloplasmin

Ceruloplasmin-mediated copper uptake represents a novel cellular process for micronutrient transport (147). Ceruloplasmin is the most abundant copper protein in plasma and contains 70–95% of plasma copper (62). The ferroxidase activity of ceruloplasmin is of uncertain physiologic significance (147), but ceruloplasmin clearly plays a role in copper transport. Each ceruloplasmin protein tightly binds 6 or 7 copper atoms to a variety of copper-binding sites (116, 147) and can contribute copper to cells (18, 67, 101) and to intracellular copper proteins (29).

Copper derived from ceruloplasmin enters the cell, but the protein does not (132). In contrast, co-uptake of protein and metal occurs in transferrin-mediated iron uptake. Sulfhydryl-modifying reagents inhibit the uptake of copper and implicate sulfur amino acids in the transport process. Inhibition of copper uptake by cuprous chelators and the stimulation of copper uptake by ascorbate suggest that copper is taken up as Cu(I) rather than as Cu(II) (61, 131).

A ceruloplasmin receptor may facilitate copper uptake by the mammalian cell. Specific ceruloplasmin-binding sites on several cell types and tissues support the notion of a ceruloplasmin receptor (8, 56, 80, 81, 121, 163). Potential receptors purified or enriched by a variety of affinity-based methods

differ in size and composition (8, 119, 161), and there is no compelling evidence supporting one putative receptor over the others. The numerous proteins identified as putative receptors may represent components of this complex, all of which exhibit some affinity for ceruloplasmin.

At least half of the copper from the ceruloplasmin first enters a membrane-enclosed space, perhaps endosomes, which copurifies with clathrin-coated vesicles (39). Radioactive copper can be tracked from this compartment and later appears in copper proteins such as superoxide dismutase 1 (SOD1) (39). The initial uncertainty regarding the role of ceruloplasmin-mediated copper transport has given way to the ability to characterize potential ceruloplasmin receptors and to define a pathway for these receptors. Such thorough biochemical characterization of ceruloplasmin-mediated copper uptake lays the groundwork for the future identification of the components responsible for this process.

Free Copper Uptake: Intestinal Mucosa Apical Transport

Entry of free copper into the cell follows a path different from that of copper bound to ceruloplasmin. Uptake by three extensively studied cell types—intestinal mucosal cells, fibroblasts, and hepatic cells—is considered separately. Although the focus of study and the specifics of transport differ in each system, the similarities are sufficient to support a unified view that copper uptake involves mechanisms common to all cell types.

Studies of combined apical, intracellular, and basolateral copper transport as well as studies that measure isolated apical copper transport from the intestinal lumen into the intestinal mucosa show mammalian intestinal copper transport to be a saturable, presumably carrier-mediated, process (27, 175) with at least one saturable transport component (175). Intestinal luminal contents, such as amino acids (182), ascorbic acid (177), fiber, and ethylenediaminetetraacetate (EDTA) (174), affect combined intestinal copper uptake in various ways. Studies that focus on isolated apical transport provide a clearer view of the process and demonstrate copper uptake kinetics consistent with a mediated process at physiologic concentrations (12, 183), with possible exceptions (174). Both zinc (47) and cadmium (40) inhibit combined intestinal copper uptake; one component of this inhibition may be a direct block of apical copper transport. High luminal zinc concentrations result in immediate inhibition of copper transport (118). One component of zinc inhibition of intestinal copper uptake may therefore be direct interference with an apical copper transporter.

Free Copper Uptake: Hepatocytes and Fibroblasts

Although some differences may be apparent between the copper uptake of cultured hepatic cells and that of fibroblasts, several striking features are shared

across species and cell types. As in ceruloplasmin-mediated transport, extracellular ligands do not enter the cell with copper (43, 176). Most studies support an energy-independent, saturable, presumably carrier-mediated process (43, 104, 154, 164, 185), although some do not (106). Inhibition of uptake by other metals, such as zinc or cadmium, suggests the existence of a general metal-ion carrier rather than a copper-specific carrier (43, 154). In studies that examined intracellular distribution of transported copper immediately after transport, significant amounts of copper were found in both cytoplasm and membrane-bound fractions in both hepatocytes and fibroblasts (154, 180).

Histidine and albumin have different effects on copper uptake. Histidine-bound copper comprises a significant fraction of circulating copper, whereas albumin is a major copper-binding protein in the portal circulation (62, 186). Histidine at ratios less than or equal to 1:1 with copper do not affect copper transport. At higher concentrations, histidine acts as a weak competitive inhibitor of transport in hepatocytes (35) and as a noncompetitive inhibitor in fibroblasts (180). Histidine facilitates copper uptake when albumin is present and has been proposed as a physiologic carrier of copper in plasma for both hepatic and nonhepatic cells (35, 104).

Copper initially bound to albumin may enter cells more slowly than unbound copper or copper bound to other ligands (35, 43, 176, 181). However, overall uptake of copper by hepatocytes in the presence of albumin may be higher than that of other cell types, possibly owing to an additional hepatocyte intracellular copper-binding protein (181). Albumin binds copper predominantly via an N-terminal histidine-rich region (138). This site may be critical for facilitation of uptake, as copper bound to this site enters the cell more quickly than copper bound elsewhere to albumin (105). Analbuminemia in humans (184) and animals (166, 178) without defects in copper uptake argues against an essential role for albumin in copper uptake. The physiologic role of albumin in the mediation of copper transport therefore remains controversial and requires further study.

Conclusions

A composite view of free copper transport into the cell emerges from the study of the different cell types. All are carrier mediated and not dependent on cellular energy. Zinc or other metals likely compete directly with copper for uptake. Extracellular ligands have variable effects, which may reflect experimental or cell type–specific differences. The similar parameters of noncerulo-plasmin copper uptake in the different cell types suggest that a single uptake process and common transport proteins are used by all cell types.

Copper routed through the two uptake systems, ceruloplasmin-mediated and free copper uptake, at some point converges into a common path. Most nonhepatic cells likely possess both pathways. In vivo and in vitro studies dem-

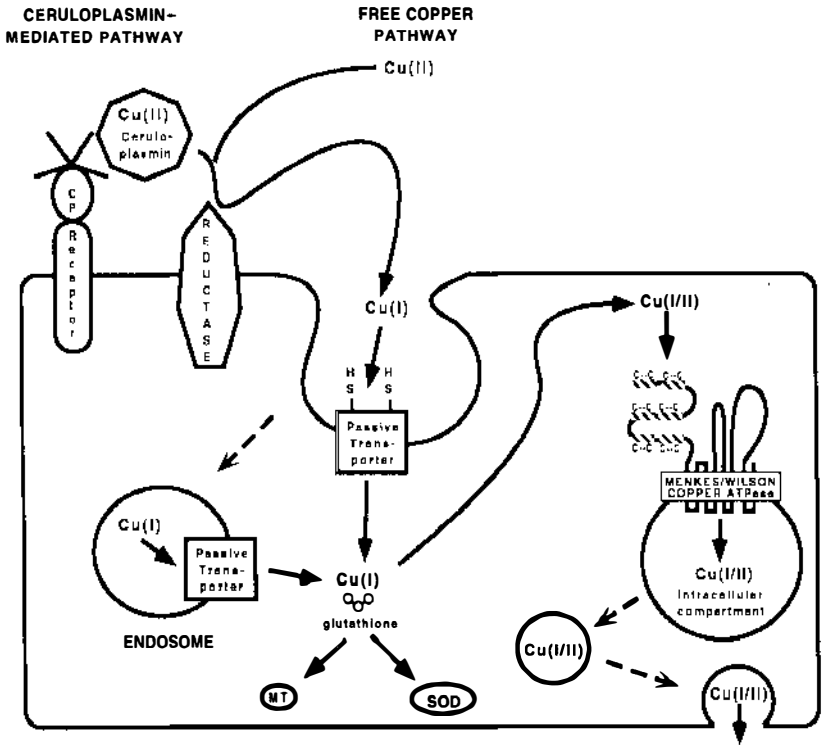

Figure 3 A unified model of copper transport. The model indicates that both ceruloplasmin-mediated and free copper transport utilize the same cellular components in the uptake process. In ceruloplasmin-mediated transport, binding to a ceruloplasmin receptor is followed by release of copper from ceruloplasmin in a step requiring copper reduction. Cu(I) enters the cytoplasm via a passive transport protein that does not require cellular energy to function. Some copper transport into the cytosol occurs at the cell surface, and some occurs after internalization of the copper-transporter complex in an endosome. Cu(I)-GSH transfers cytosolic copper to cuproproteins, such as MT or superoxide dismutase (SOD). Copper is delivered to the export system by the Menkes or Wilson copper-transporting ATPase in one or more intracellular compartments or organelles. In this model, free copper or copper bound to other ligands utilizes the same reductase and follows the same path as copper from ceruloplasmin.

onstrated that albumin, histidine, or ceruloplasmin can deliver copper to copper proteins such as SOD1 in many of the same cell types (29, 55). Several intriguing studies demonstrate an interaction between the two pathways, perhaps at the copper entry phase. In one study, free copper inhibited uptake of copper from ceruloplasmin (121). The converse, inhibition of free copper uptake in the presence of ceruloplasmin, was noted in another study on placental copper transport (107). A further study found similar rates of copper uptake of free copper and of copper bound to ceruloplasmin (102). The eluci-

dation of the relationship between the two will likely help clarify the individual mechanisms involved.

We present a unified model of copper uptake in Figure 3. In this model, a ceruloplasmin receptor brings ceruloplasmin to the cell surface, where copper is released by reduction from ceruloplasmin. The copper is then captured by the as-of-yet unidentified passive copper carrier protein. Endocytosis brings the copper initially into the cell within endosomes, from which it then exits into the cytoplasm via the copper carrier. Free copper or copper bound to other ligands utilizes the same reductase and follows the same path as copper from ceruloplasmin. The interface of these uptake systems with subsequent steps of intracellular copper transport remains unexplored and is the next frontier for investigation.

INTRACELLULAR COPPER DISTRIBUTION

Copper and copper proteins are distributed throughout the cell and in all cellular organelles, including the nucleus, mitochondria, lysosomes, endoplasmic reticulum, and cytosol (99). One fourth to one half of cellular copper is cytosolic. Slightly less copper is present in the nucleus, and a smaller but nonetheless significant amount is found in the mitochondria and endoplasmic reticulum. Copper proteins are similarly located in all cellular compartments. Examples include SOD1 in the cytosol and (perhaps) peroxisomes (28); cytochrome *c* oxidase in mitochondria (84); lysyl oxidase in the Golgi and secretory organelles (86); and MT in the cytosol, nucleus, and lysosomes (70, 71).

The means by which the cell delivers copper to cuproproteins, regulates intracellular copper levels, and compartmentalizes copper are poorly understood. MTs had been proposed for multiple roles in copper distribution, regulation, and delivery. However, recent work questions whether MTs play a central role in copper distribution and instead supports a primary role for MTs in detoxification, either directly through copper sequestration or through SOD-like activity of the copper-bound MT (168).

Experimental and clinical investigations suggest that MTs do not play a role in the delivery of copper to proteins responsible for copper export. Zinc, which directly inhibits intestinal apical uptake of copper (see above), also induces MT synthesis in intestinal mucosal cells after prolonged exposure (142, 151). In intestinal mucosal cells of animals fed diets high in zinc, copper uptake is reduced and more copper is bound to MT, but basolateral copper transfer across the serosal surface is unaffected (47, 48). A clinical study similarly showed increased intestinal MT levels and decreased copper absorption after zinc treatment (193). Because copper has a higher affinity for MT than does zinc (41), it has been proposed that copper displaces zinc from MT (58). Hence, MT may trap copper that enters the cell and divert that copper from normal

basolateral export pathways. It follows that intracellular copper transit from the apical transporter to the basolateral transporter is unlikely to occur via MT under normal physiologic conditions.

The generation and breeding of mice simultaneously inactivated for *MT-I* and *MT-II* genes provide a direct test of the role of MT in copper metabolism (103, 113). The double knockout mice are liveborn, have no detected developmental abnormalities, and reproduce normally. Because inherited or environmental copper deficiency results in multiple developmental defects, either these MTs play no essential role in delivery of copper to cellular enzymes, or redundant systems such as other MT isoforms (130, 137, 160) compensate for MT-I and MT-II deficiency. The knockout mice do show an increased sensitivity to cadmium toxicity, indicating that MT may provide protection from excess levels of at least one metal ion (103).

GSH and not MT may mediate copper incorporation into nascent cytosolic apoproteins requiring copper. It may also contribute to cellular defenses against copper toxicity. High levels of copper GSH were thought to mediate copper resistance in copper-selected hepatoma cell lines (50), although some investigators have expressed reservations (80). Virtually 100% in vitro reconstitution of Cu-MT and Cu-SOD from copper-free apoproteins and Cu(I)-GSH is consistent with a role for GSH in copper delivery (5, 24, 46). In contrast, in vitro reconstitution studies of apo-SOD by Cu-MT found limited reactivation dependent on nonphysiologic oxidation of MT (52). Although further work is required to confirm the in vivo function of GSH, GSH depletion prevented copper delivery to apo-SOD (159) and apo-MT (158). Other cytosolic components, such as the 38-kDa and 50-kDa copper-binding proteins isolated from hepatic cytosols, may also contribute to the intracellular copper distribution (128).

Although transport of copper into membrane organelles is poorly understood, lysosomal transport is clearly a critical step in copper disposition. Hepatic copper overload results in a disproportionate increase in lysosomal copper (88, 188), which may be bound to MT. In mutant beige mice with abnormal lysosomes, hepatic lesions develop rapidly in response to a copper load (63). We have already indicated (see above) that yeast with defective vacuoles (lysosome equivalents) are hypersensitive to copper (42). The lysosome therefore may be a depot for excess copper and may assist in the management of copper stress.

Other disorders of copper metabolism, such as toxic milk in the mouse, may help us identify critical components of cellular copper transport. Pups born to and nursed by toxic-milk homozygotes exhibit curly whiskers, hypopigmentation, stunted growth, abnormal motor behavior, and tremors, and they die as neonates (140). Both heterozygous and homozygous pups born to homozygous mothers are affected, and the disease manifestations result from an autosomal

recessive disorder of copper metabolism in the mother. Pups are born copper deficient, and the milk of homozygous females is copper deficient. Copper treatment or cross-fostering to a normal lactating female restores the copper status of the pups and enables them to develop normally. The toxic-milk females therefore reflect a defect of copper transfer into both the placenta and the milk.

Both male and female homozygous toxic-milk adult mice accumulate excessive copper in the liver and have depressed ceruloplasmin levels (10). As in Wilson disease, increased liver copper initially accumulates diffusely in the cytoplasm bound to MT in toxic-milk mice (141), with lysosomal accumulation occurring in later stages of the disease (66). However, the cytopathology of hepatic copper accumulation in toxic milk differs from that observed in Wilson disease (10, 66) and suggests a different etiology. The role of MT in toxic milk is controversial. In one study, increased MT mRNA expression was thought to be a secondary response (111), whereas other investigators found reduced MT mRNA expression but increased MT protein stability (85). Further study is clearly warranted, not only to elucidate the basis of this disorder, but also to understand the tissue specificity involved.

A combination of environmental and genetic factors may result in Indian childhood cirrhosis (ICC) (4, 34, 73). Children with ICC accumulate massive amounts of copper in the liver and develop cirrhosis and liver failure. Until recently, patients invariably died in infancy. Now, however, the disorder can be treated with D-penicillamine (9). Serum ceruloplasmin levels are normal in affected individuals (73). Copper accumulates in the cytoplasm, possibly as copper-sulfur-protein complexes (2, 9). The liver of an ICC patient exhibits a characteristic pathology distinct from that of other liver diseases, including Wilson disease (73).

The use of copper cooking utensils in Indian households and the high copper levels in milk boiled in such vessels have led some investigators to suggest an environmental etiology for the disorder (120). However, segregation analysis of 120 families with ICC indicated an autosomal recessive inheritance pattern (2). In five non-Indian families with children with ICC, no source of increased copper intake was found (3, 65, 90, 100). These included two different American families with multiple affected siblings, with consanguinity in one of the families (1). A recent examination of ICC patients in India found that in 46% of cases, the use of copper vessels in food preparation was denied, indicating that dietary copper was not the causative agent (155).

Biochemical abnormalities in cell lines from American patients with ICC strengthened the argument for a genetic etiology and provided the first molecular information on ICC (57). Although cultured fibroblasts from patients with ICC exhibited normal copper uptake and levels, these cells had disturbed histology and cellular response to metal stress. Dilated rough endoplasmic

reticulum, vesicular aggregates, and fibrillar whorls (reminiscent of the liver pathology in ICC) were evident in the fibroblasts, suggesting a systemic rather than a liver-specific defect. Even more striking was the observation that basal and metal-induced MT mRNA and MT protein synthesis were severely depressed. Induction of MT by copper, zinc, and cadmium was defective, whereas MT induction by glucocorticoid hormone was normal. MT protein turnover was normal, and no mutations were detected in the hMTIIa coding region or promoter. This work suggests a genetic defect in MT synthesis in response to metals, perhaps in a metal-responsive metalloregulatory protein. Although there is now strong evidence for a genetic etiology in at least some cases of ICC, we cannot rule out a potentiating role for dietary copper or even the existence of two distinct disorders, one with a genetic origin and the other of environmental etiology (65).

RELEASE OF COPPER FROM CELLS

Menkes Disease

Two human inherited disorders illustrate the importance of release of copper from cells. In autosomal recessive Wilson disease and in the animal model for Wilson disease, the LEC rat, decreased liver copper export results in copper-induced chronic liver disease and pathologic changes in the brain, kidney, and eye (33). The features of Wilson disease reflect the effects of copper toxicity. In contrast, in X-linked Menkes disease and the corresponding animal model, the mottled mouse, defective copper export causes trapping of copper in some tissues (notably intestinal mucosa and kidney), leading to failure of copper delivery to other tissues. The clinical features of Menkes disease therefore largely result from a systemic copper insufficiency (33).

Patients with Menkes disease show pili torti, hypopigmentation, growth failure, skeletal defects, arterial aneurysms, hypothermia, seizures, and progressive degeneration of the central nervous system, with death occurring in early childhood (109). Hemizygous male mottled mice can exhibit many of these same pathologic findings (68, 114). Deficiencies in cuproenzymes in multiple tissues—e.g. tyrosinase (hypopigmentation), lysyl oxidase (defective collagen, elastin cross-linking), and dopamine-β-hydroxylase (catecholamine production)—are likely responsible for some of the features of this disease (32, 135).

Defective copper export is the basic cellular defect in Menkes disease and the mottled mouse. With the exception of hepatocytes (36) and cultured chorionic villus cells (170), all tested Menkes and mottled cultured cells exhibit excessive copper accumulation (33). Menkes and mottled cells have a specific defect in copper efflux, with normal uptake, and with normal transport of

cadmium and zinc in mutant cells under experimental conditions (64, 124, 125). Increased concentrations of MT protein and mRNA have been demonstrated in Menkes and mottled cells (33, 83, 88a, 91, 122, 126), but regulation of MT synthesis is normal (91, 122, 126), suggesting that increased MT is a secondary effect.

The Menkes and Mottled Genes

Three independent groups (23, 112, 179) recently isolated the gene responsible for Menkes disease. The gene was identified by a positional cloning approach, based on gene localization to the Xq13 region of the human X chromosome by analysis of linkage (171) and chromosomal rearrangements (79, 172, 173). The gene was expressed in all tissues tested except liver, a pattern consistent with the Menkes phenotype. Sequence analysis of the *MNK* cDNA and the predicted 1500–amino acid protein revealed that the Menkes locus likely encodes a copper-transporting P-type ATPase (179). Diverse mutations in the *MNK* gene result in abnormal expression of the gene (23, 38, 112, 179). Approximately 16% of patients with severe Menkes disease had partial, nonoverlapping deletions of the *MNK* gene (23, 112, 179). In patients without large gene deletions, the independently occurring mutations detected in the *MNK* gene were different in each family and were predicted to have a severe effect on the structure or expression of the Menkes protein (38). Results to date indicate that mutations unique to each family are responsible for the vast majority of cases of Menkes disease.

The *Mo* locus was mapped on the mouse X chromosome near the phosphoglycerate kinase (*Pgk1*) locus (11), a region homologous to the human Xq13 region containing the *MNK* gene. The mouse homologue of the *MNK* gene was cloned (94, 110). This homologue was shown to map near *Pgk1* and was expressed in the same tissues as the *MNK* gene. In a severe mottled mutant (Mo^{dp}), the mouse gene was not expressed in the hemizygote, but the exact molecular lesion in the *Mo* gene responsible for Mo^{dp} remains to be defined (53). In a second, milder mutant (Mo^{blo}), abnormal expression of the gene (37, 94, 110) occurred as a result of a splice-site mutant in the Mo^{blo} gene. These data suggest that the mouse homologue of the *MNK* gene is the *Mo* gene and that the mottled mouse is the murine model for Menkes disease.

Milder variants of Menkes disease and mottled mouse mutants may provide insight into the function and intracellular location of the Menkes and mottled copper-transporting ATPase. One such variant is the occipital horn syndrome (OHS), a connective tissue disorder characterized by hyperelastic skin; bladder diverticulae; and skeletal abnormalities, including bony exostoses of the occiput (89). The disease is sometimes accompanied by mild neurologic impairment, in contrast to the severe neurologic degeneration of Menkes disease.

OHS is likely an allelic variant of Menkes disease. In both diseases, intestinal

absorption of copper is deficient, serum copper and ceruloplasmin are low, and cultured fibroblasts accumulate copper (87). In addition, several of the connective tissue manifestations are similar in the two disorders. The *Mo^{blo}* mouse exhibits abnormalities reminiscent of those seen in OHS (114), indicating that at least one mouse mutant has a connective tissue phenotype similar to OHS. The clinical features of OHS can be related to a secondary deficiency of lysyl oxidase (17, 87), which is encoded by a gene on human chromosome 5 (59).

A preliminary analysis revealed that *MNK* mRNA was greatly reduced in fibroblasts from two unrelated patients with OHS (93). Two independent groups (37, 76) detected mutations in OHS that affect the efficiency of normal splicing. Splice-donor or acceptor-site mutations resulted in the expression not only of an abnormal mRNA (due to exon skipping), but also of some quantity of normally spliced message. Analysis of *Mo^{blo}* (37) revealed a splice-donor mutation that results in both abnormal exon-deleted transcripts and mRNA of normal sequence. Both groups (37, 76) suggested that such subtle splicing mutations permit the synthesis of a small amount of normal copper-transporting P-type ATPase protein in OHS and other mild variants.

The primarily connective tissue phenotype of OHS and *Mo^{blo}*, in the presence of some postulated amount of normally functioning Menkes or mottled transport protein, suggests that lysyl oxidase may be particularly sensitive to defects in the activity of the Menkes and mottled copper-transporting ATPase. In contrast, tyrosinase and cytochrome *c* oxidase do not exhibit such sensitivity (69, 108, 123, 146). It has been suggested (37) that the sensitivity of lysyl oxidase, which, unlike other cuproenzymes, is secreted, could reflect a requirement for the Menkes and mottled copper-transporting ATPase in the transfer of copper to the specific cell compartment in which hololysyl oxidase is formed. Other cuproenzymes may be located in cellular compartments more accessible to available copper, in which case an additional transport step mediated by the Menkes and mottled mouse proteins would not be required.

Copper efflux and cell fractionation studies are consistent with the notion that the copper accumulation of Menkes cells results from a defect in copper translocation across a subcellular compartment rather than from a plasma-membrane defect in copper export (33, 64, 123, 127). Other data suggest defective entry of copper into a number of cell compartments. Decreased copper content in mitochondria and lysosomes has been directly demonstrated in mutant cells by biochemical measurements (83), electron microscopic cytochemistry (83), and cell fractionation experiments (127). The biochemical data and mutant phenotypes suggest that the Menkes and mottled gene products may be important for copper delivery to mitochondria and lysosomes as well as for copper delivery to secretory path organelles such as the endoplasmic reticulum or the Golgi. Given the copper export defect in Menkes and mottled

cells, we propose that the normal pathway of copper export may well include one of these intracellular compartments (Figure 3).

Wilson Disease

Wilson disease is an autosomal recessive disorder classically characterized by the coexistence of progressive neurologic findings, chronic liver disease with cirrhosis, renal tubular dysfunction, and pigmented corneal rings (Kayser-Fleischer rings). Neurologic symptoms may include behavior disturbances, dysarthria, and movement disorders. The age of onset ranges from 4 to 50 years, with an earlier age of onset generally associated with hepatic disease, often without neurologic manifestations. The copper content of liver, brain, kidney, and cornea is increased (162).

Impaired intracellular transport of copper results in decreased biliary excretion of copper and reduced incorporation of copper into ceruloplasmin. Ceruloplasmin levels and total serum copper concentrations are decreased in most patients, whether measured by enzymatic activity or immunochemical methods (98, 162). Normally, copper appears to be incorporated into ceruloplasmin in the rough endoplasmic reticulum (RER) immediately following ceruloplasmin synthesis, in a step independent of ceruloplasmin secretion (54, 150). Wilson disease leads to a perturbation in the incorporation of copper into ceruloplasmin and/or in ceruloplasmin secretion, perhaps owing to defective copper transport into the organelles of the secretory pathway.

In the early stages of Wilson disease, copper accumulates diffusely in the cytoplasm (4) bound to MT. Later in the disease, copper is sequestered as copper-associated protein in lysosomes (4). In contrast, in hepatic copper overload resulting from other causes (biliary atresia, nutritional overdose), copper first increases rapidly and disproportionately in the lysosome (4, 162). It has therefore been proposed that the biliary secretion defect in Wilson disease may be related to the failure of transport of copper into lysosomes (51, 162). Both Menkes disease and Wilson disease apparently can result in defects of a secreted protein (lysyl oxidase and ceruloplasmin, respectively) as well as in defects in copper transport into the lysosome. Analogous transport steps in the RER, lysosomes, and/or other organelles may therefore be mediated by homologous proteins in liver (Wilson disease) and other tissues (Menkes disease).

Studies of autosomal recessive copper toxicosis in the LEC rat revealed parallels to Wilson disease (115, 153), including copper excess in liver and kidney, low serum ceruloplasm (95), decreased biliary excretion (167), and increased copper in certain sectors of brain (165). The early failure of transfer of copper from a cytosolic to a noncytosolic compartment in liver (189) is reminiscent of the anomalous cellular distribution in human Wilson disease. Finally, hepatitis can be prevented by treatment with D-penicillamine, a copper-chelating agent used to treat Wilson disease (162). Copper toxicosis in a

second proposed animal model, the Bedlington terrier (72), differs from Wilson disease in its handling of hepatic copper (162) and likely maps to a different genetic locus (193a).

Molecular Genetics of Wilson Disease

The gene for Wilson disease maps to the long arm of chromosome 13 (45). The *WND* gene was independently identified by three groups of investigators (14, 15, 133, 169, 190), based on positional cloning strategies and on the hypothesis (179) that the Wilson disease protein might bear similarities to the Menkes disease P-type ATPase. *WND* cDNA expression is limited to liver, kidney, and placenta, in contrast to the widespread expression of the *MNK* gene. The *WND* gene encodes a 1411–amino acid P-type cation-transporting ATPase (15, 169), with 56% overall identity to the *MNK* gene product (14, 134). The rat homologue of the *WND* gene has also been isolated, and deficient expression due to a partial genomic deletion has been demonstrated in LEC rat liver (149, 187, 191).

No large gene deletions are evident in Wilson disease patients; rather, a predominance of single base changes or small deletions, causing frameshifts and protein truncation, has been observed (15, 133, 169). Importantly, unrelated patients were homozygous for an identical mutation (15). In a related finding, two different disease mutations were observed in linkage disequilibrium with microsatellite marker haplotypes that represented common disease-associated haplotypes in American and Russian populations (133, 169). A few mutations therefore appear to account for a significant fraction of cases in specific populations, and such epidemiologic correlates should find application in clinical molecular diagnosis. This pattern differs from that of Menkes disease (23, 37, 112, 179), in which large deletions account for a significant fraction of mutations. Moreover, every family afflicted with Menkes disease to date has had a different mutation.

Analysis of *WND* mRNA revealed that the brain (and possibly kidney and placenta) contains transcripts with several different combinations of skipped exons (134, 169). One of these mRNAs results in a truncated protein, and another lacks the transmembrane domain containing the Cys-Pro-Cys motif, which is thought to be essential for cation transport (134). It has been speculated that such alternative splicing could be a regulatory mechanism, or that it could result in a gene product with a different function from that of the liver protein (134). These speculations are intriguing because of the involvement of other tissues in Wilson disease (162) and because of the disassociation of hepatic and neurologic symptomology in the LEC rat (115, 153, 165) and, in some studies of the effects of liver transplant, in humans (152).

P-Type ATPases

The *MNK* and *WDN* genes and their respective animal homologues encode copper-transporting P-type ATPases (14, 15, 94, 110, 169, 187). P-type ATPases transport cations using ATP and form a family of integral membrane proteins in bacteria, fungi, plants, and animals. This family of more than 70 members includes Ca^{2+}, $Cu^{+/2+}$, Cd^{2+}, H^+, K^+, Mg^{2+}, H^+/K^+, and Na^+/K^+ transport ATPases (19). All members have a molecular weight greater than 100,000, with between 6 and 10 transmembrane domains (7), and likely function as monomers to pump ions through a membrane. P-type ATPases form subfamilies according to the ion transported. For instance, the human H^+ transporter is more closely related to other H^+ transporters than to human Ca^{++} transporters (44), and copper-transporting ATPases are more closely related to each other than to other P-type ATPases. The *MNK* and *WND* gene products and their respective homologues bear the greatest similarity to the family of known and proposed copper-transporting ATPases.

A model of the Menkes and Wilson copper-transporting ATPases (15, 134, 179) is presented in Figure 4. Copper is proposed to bind initially to the N-terminus of the protein via six metal-binding domains. Copper ions are then passed to the P-type ATPase core. Phosphorylation of an invariant aspartate by an ATP bound to the ATP-binding/kinase domain followed by dephosphorylation of the same residue by the phosphatase domain results in conformational changes in the protein, transferring probably one or two cations onto the other side of the membrane. For some P-type ATPases, such as Na^+/K^+ ATPase, counter ions traverse in opposite directions. Unidentified counter ions may exist for copper.

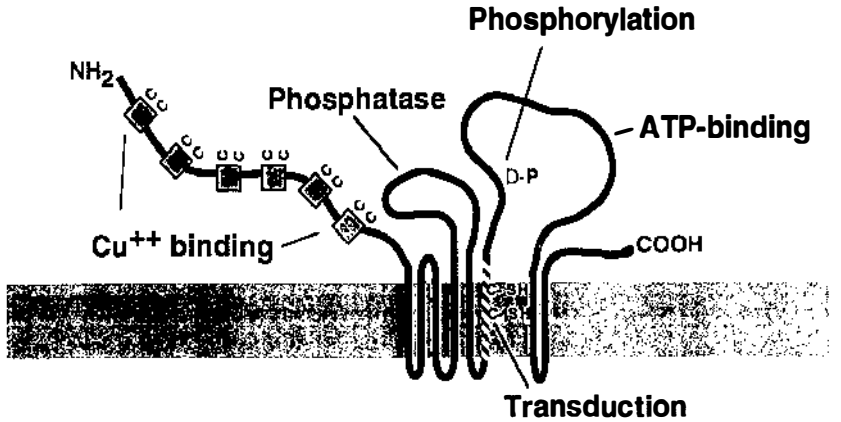

Figure 4 Model for the proposed Menkes and Wilson copper-transporting ATPases based on homology to other P-type ATPases. The boxes with the paired cysteines (C) represent the metal-binding motifs. Adapted with permission from Reference 179.

Genetic and biochemical studies indicate that the related proteins described above are involved in copper transport, although direct biochemical demonstration of copper transport is lacking. The *Enterococcus hirae* CopA and CopB proteins were the first described and most extensively characterized members of this group of proteins (157). Phenotypes of *E. hirae* individually deleted for the *copA* and *copB* genes support a role for the CopA protein in copper uptake and for the CopB protein in copper export (117). Disruption of the *ctaA* gene in *Synecocchus 7942* results in increased copper tolerance, and the CtaA protein is proposed to import copper (136). In contrast, deletion of the *pacS,* an additional *Synecocchus 7942* ATPase, results in copper hypersensitivity. PacS is located in the membrane of the thykaloid (similar to a chloroplast) of the bacterium and has been hypothesized to export copper from the organelle (77, 78). Additional copper-transporting ATPases have been described in *S. cervisiae* (139), *Rhizobium mellioti* (75), and other microbial species.

One striking feature of the copper- and other metal-transporting ATPases (156) is the presence of potential copper-binding motifs in the N-terminus of the proteins. The N-termini diverge among families of P-type ATPases of different ion specificity. The N-termini of most of the copper-transporting ATPases are related, and all contain potential copper-binding motifs. These motifs in the copper-transporting ATPases are similar to a metal-binding motif originally defined in mercury detoxification proteins and cadmium transporters and consist of a highly conserved core of approximately 30 amino acids within an extended 72–amino acid motif (134). A pair of cysteine residues in the motif, which has been shown to be critical for metal binding in a mercury protein (148), likely plays a role in copper binding. The Menkes, mottled, and Wilson P-type ATPases each contain six of these motifs; the *LEC* gene contains five; the yeast homologue, *Ccc2,* contains two; and the remainder of P-type ATPases each contain one. The domains in the copper-transporting ATPase exhibit greater similarity to each other than to the motifs in the mercury proteins and cadmium transporters, suggesting an intrinsic ion specificity (156). The *E. hirae* CopB protein does not contain one of these motifs but instead contains an alternative metal-binding motif homologous to the methionine-rich motif in *P. syringae* CopA and CopB (117).

OVERVIEW AND CONCLUSIONS

Common themes in copper transport in unicellular and mammalian organisms discussed in this review reflect the requirements for copper in basic cell biochemical processes as well as the toxicity of copper excess. Among the diverse organisms, examples of protein components involved in copper trans-

port, uptake, intracellular distribution, and export have been identified. Whether in bacteria, yeast, or mammalian cells, copper import requires the coordinate function of several proteins with both metal-binding and catalytic domains in a mediated transport step. In at least *S. cerevisiae* and mammalian cells, these components appear to function in the transport of additional metals, such as iron, or they can be affected directly by other metals, such as zinc. The potential reactive properties of copper require intracellular mechanisms for copper delivery to cuproproteins and for detoxification. GSH, rather than MT, may fulfill the cytosolic copper delivery function in mammals. Additional proteins play a role in bacteria and suggest that a more complex picture may emerge in mammalian cells. Cells across species deal with excess copper in one of three ways: by binding it to specific detoxification proteins, such as mammalian or *S. cerevisiae* MTs and *P. syringae* CopA; by transporting copper into an isolated cellular compartment, such as the lysosome, vacuole (*S. cerevisiae*), or periplasm (*P. syringae*); or by exporting it out of the cell. *S. cerevisiae* and bacterial copper transport proteins are likely to have functional homologues in mammalian systems. Individual domains of these proteins, e.g. metal-binding, reductase, and ATPase domains, are likely to have structural homologues among species. Exact function may differ depending on the needs of the different cell types and the extracellular environment of the cell. A striking example is provided by the evolutionarily conserved copper-transporting ATPases, such as the *MNK* and *WND* gene products and the *E. hirae* CopA protein, which export copper from cells either directly or via intracellular compartments. We have attempted in this review to integrate the knowledge of copper transport in different organisms, to highlight the commonalities, to underscore the gaps in knowledge, and to provide paradigms for future research.

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