Critical Review

A Delicate Balance: Homeostatic Control of Copper Uptake and Distribution[1]

Maria M. O. Peña, Jaekwon Lee and Dennis J. Thiele[2]

Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, MI 48109-0606

ABSTRACT The cellular uptake and intracellular distribution of the essential but highly toxic nutrient, copper, is a precisely orchestrated process. Copper homeostasis is coordinated by several proteins to ensure that it is delivered to specific subcellular compartments and copper-requiring proteins without releasing free copper ions that will cause damage to cellular components. Genetic studies in prokaryotic organisms and yeast have identified membrane-associated proteins that mediate the uptake or export of copper from cells. Within cells, small cytosolic proteins, called copper chaperones, have been identified that bind copper ions and deliver them to specific compartments and copper-requiring proteins. The identification of mammalian homologues of these proteins reveal a remarkable structural and functional conservation of copper metabolism between bacteria, yeast and humans. Furthermore, studies on the function and localization of the products of the Menkes and Wilson's disease genes, which are defective in patients afflicted with these diseases, have provided valuable insight into the mechanisms of copper balance and their role in maintaining appropriate copper distribution in mammals. J. Nutr. 129: 1251–1260, 1999.

KEY WORDS: • *copper* • *transport* • *distribution* • *Menkes* • *Wilson's disease* • *homeostatic regulation*

For many decades it has been widely accepted that Copper (Cu)[3] is an essential trace element required for survival by all organisms from bacterial cells to humans (Linder 1991). What is so special about this trace element that makes it essential in biology? Copper ions undergo unique chemistry due to their ability to adopt distinct redox states, either oxidized [Cu(II)] or in the reduced state [Cu(I)]. Consequently, Cu ions serve as important catalytic cofactors in redox chemistry for proteins that carry out fundamental biological functions that are required for growth and development. As shown in **Table 1**, Cu requiring proteins are involved in a variety of biological processes and deficiency in these enzymes, or alterations in their activities, often cause disease states or pathophysiological conditions. The dietary intake of Cu by humans has been estimated to range from 0.6–1.6 mg Cu per day (Linder and Hazegh-Azam 1996), through the ingestion of food sources rich in Cu such as legumes, beef and shellfish. The Cu that is ingested is readily absorbed and distributed to copper-requiring proteins with apparently little storage of excess Cu in the body.

While it is clear that Cu is essential, it is also a potent cytotoxin when allowed to accumulate in excess of cellular needs. Again, due to the special redox chemistry of this metal ion, similar to that of iron (Fe), Cu readily participates in reactions that result in the production of highly reactive oxygen species (ROS) including hydroxyl radical (Halliwell and Gutteridge 1984). Hydroxyl radicals are believed to be responsible for devastating cellular damage that includes lipid peroxidation in membranes, direct oxidation of proteins, and cleavage of DNA and RNA molecules. Indeed, the generation and action of ROS are thought to be major contributing factors to the development of cancer, diseases of the nervous system and aging (Halliwell and Gutteridge 1990). In addition to the generation of ROS, Cu may manifest its toxicity by displacing other metal cofactors from their natural ligands in key cellular signaling proteins. For example, the replacement of Zn(II) by Cu(II) in the zinc-finger DNA binding domain of the human estrogen receptor renders this protein defective in binding to its cognate target DNA sequences in vitro, thereby potentially interfering with its role in hormone-dependent signal transduction in vivo (Predki and Sarkar 1992). It is highly likely that Cu is able to displace metal ions in a number of catalytic or structural motifs in many cellular proteins. Given that Cu is both essential and toxic, organisms must implement uptake mechanisms to extract Cu from nutrients, transport Cu across biological membranes and deliver it to Cu-requiring proteins. Furthermore, precise regulatory mechanisms must be in place to prevent the accumulation of Cu ions to toxic levels. The importance of maintaining this critical balance is underscored by the existence of two well-characterized human genetic diseases in Cu transport, Menkes and Wilson's diseases (Bull and Cox 1994, Bull et al. 1993,

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[2] To whom correspondence should be addressed

[3] Abbreviations used: CCS, copper chaperone for SOD1; CJD, Creutzfeld-Jacob disease; Cu, copper; EXAFS, x-ray absorption fine structures; Fe, iron; FFI, fatal familial insomnia; GGS, Gerstmann-Straussler-Scheinken; MNK, Menkes; PINA, pineal gland night specific ATPase; ROS, reactive oxygen species; TNG, trans-golgi network; WND, Wilson's disease.

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TABLE 1

Copper-binding proteins

Common Name	Biological Function	Consequence of Deficiency or Defect
Cu/Zn SOD	Free radical detoxification	Oxidative damage of cell components
Cytochrome c oxidase	Electron transport in the mitochondria	Symptoms of deficiency of ATP: myopathy, ataxia, seizures
Lysyl oxidase	Crosslinking of collagen and elastin	Connective tissue symptoms: vascular rupture and torsion Loose skin and joints, emphysema
Dopamine β-hydroxylase	Catecholamine production	Hypothalamic imbalance : hypothermia, hypotension, dehydration, somnolence
Tyrosinase	Melanin production	Depigmentation
Peptidylglycine Monooxygenase	Bioactivation of Peptide hormones	Probable widespread effects through malfunction of several peptide hormones
Ceruloplasmin	Ferroxidase, Cu transport	Anemia
Clotting Factor, V, VIII	Blood clotting	Bleeding tendency
Angiogenin	Induction of blood vessel formation	Defective blood vessel development
Metallothionein	Cu-sequestration	Cu toxicity
Prion protein	Normal function currently unknown; Copper binding properties suggests potential role in Cu uptake	Altered sleep patterns and circadian rhythm in mice, Creutzfeld Jacob disease, Kuru, Gerstmann-Straussler-Scheinker disease, Fatal familial insomnia
β-amyloid precursor protein	Normal function currently unknown	Familial Alzheimer's Disease
Hephaestin	Iron egress from intestines	Sex-linked anemia

Chelly et al. 1993, Mercer et al. 1993, Vulpe et al. 1993, Yamaguchi et al. 1993). The identification of the human genes that are defective in these, and perhaps other Cu metabolic diseases, and the characterization of their gene products will greatly enhanced our understanding of Cu ion homeostasis in general.

Maintaining appropriate Cu homeostasis demands a critical orchestration between Cu uptake and distribution within cells, and detoxification and removal. In this review, we focus on very recent advances in the molecular mechanisms of Cu uptake into and its distribution within cells as well as its distribution to different tissues in the context of the whole organism. We also discuss the modes by which these steps are regulated in order to ensure that sufficient Cu is available to drive essential biochemical processes while preventing its accumulation to toxic levels. The reader is referred to several excellent recent reviews which emphasize different aspects of Cu metabolism including Cu detoxification (Koch et al. 1997, Winge 1998, Winge et al. 1998), Cu coordination chemistry (Koch et al. 1997), cellular Cu transport (Eide 1998, Radisky and Kaplan 1999, Vulpe and Packman 1995), nutrition (Linder and Hazegh-Azam 1996), links with iron transport (Askwith and Kaplan 1998, Winzerling and Law 1997), and Menkes and Wilson's diseases (Bull and Cox 1994, DiDonato and Sarkar 1997).

Copper uptake and absorption

Copper uptake in bacteria and yeast. Initial insight into the identification of cellular components that play key roles in Cu ion homeostasis, as for many cellular processes, was derived from studies in model systems such as bacteria and yeast. Although not necessarily entirely conserved in their mechanisms between species, a striking observation is that proteins involved in Cu ion transport share what may be considered modular functional domains. The identification of Cu transport proteins in model systems has often lead to the discovery of their mammalian homologues, or mechanistic insight into how these proteins function overall in Cu ion transport.

Recent investigations into Cu uptake in the gram-positive bacterium, *Enterococcus hirae,* have provided an excellent fundamental description of this process. In *E. hirae,* an operon containing five genes, *cop*X, Y, Z, A and B, plays an important role in Cu ion homeostasis (Odermatt et al. 1992, Solioz et al. 1994). The CopA and CopB proteins are two integral membrane P-type ATPases that are necessary for the transport of Cu into cells under Cu limiting conditions, and Cu efflux under conditions of high Cu ion levels, respectively (Odermatt et al. 1993, Odermatt et al. 1994). CopB is the only Cu transporter to date that has been biochemically demonstrated to drive the accumulation of Cu(I), and the chemically similar metal Ag(I), into reconstituted native inside-out membrane vesicles (Solioz and Odermatt 1995). Consistent with this biochemical function, disruption of the *CopB* gene renders *E. hirae* cells hypersensitive to Cu, underscoring its role in Cu extrusion during Cu overload. The CopB protein harbors three repeats of the putative metal binding motif MXHXXMSGMSHS in its amino terminus, a motif also found in a periplasmic copper-binding protein of *Pseudomonas syringae* (Cha and Cooksey 1991). CopA has a single copy of the metal binding consensus motif GMXCXXC which is found in several metal binding proteins and is now known to be involved in Cu ion coordination in proteins involved in Cu ion transport and distribution (see below). Disruption of the *Cop*A gene has no significant effect on Cu resistance in *E. hirae,* however, consistent with an important role in Cu uptake, cells with a deletion of the *CopA* gene cease to grow after three days in the presence of a Cu ion chelator, presumably due to inability to transport Cu into the cells under conditions of Cu starvation (Odermatt et al. 1993). These pioneering studies conducted in prokaryotic organisms have provided valuable insight into the molecular mechanisms by which Cu is transported in a vectorial manner, and has laid the groundwork for identifying and understanding functional homologues in eukaryotic cells.

In eukaryotic organisms, Cu ion transport and intracellular distribution is understood in greatest detail in the baker's yeast, *Saccharomyces cerevisiae.* This is in large part due to the

ease with which genetics can be used to isolate and study Cu ion transport mutants, the facile molecular biology of *S. cerevisiae*, and the fact that the entire genome of this yeast has been sequenced. **Figure 1** summarizes our current understanding of the yeast model for Cu uptake, distribution and detoxification. With the exception of the Cu-metalloregulatory transcription factors Ace1 and Mac1 (reviewed in Koch et al. 1997, Winge 1998), human homologues for yeast genes that are known to be involved in Cu ion homeostasis have been identified, underscoring the power of yeast as a model organism for understanding Cu metabolism and the strong conservation of Cu homeostatic mechanisms in virtually all eukaryotes.

In *S. cerevisiae*, high affinity Cu ion uptake has been characterized in physiological studies as a temperature and an ATP-dependent process (Lin and Kosman 1990). Studies using Cu ion electrode measurements in yeast suggest that Cu ion uptake appears to be coupled with K^+ efflux with a 1:2 stoichiometry, suggesting that this process may take place via a $Cu^+/2K^+$ antiport mechanism (De Rome and Gadd 1987). Although the exact mechanics of Cu transport into yeast cells have yet to be firmly established, recent yeast genetic studies have begun to shed light on the proteins involved in this process. Either prior to or concomitant with high affinity uptake, Cu(II) is thought to be reduced to Cu(I) by one or more cell-surface Fe(III)/Cu(II) reductases encoded by the *FRE1* through *FRE7* genes. (Georgatsou et al. 1997, Hassett and Kosman 1995) (Fig. 1). Four of these FRE genes, *FRE2-FRE6,* are transcriptionally regulated by Fe through the Fe-responsive transcription factor, Aft1, while Cu transcriptionally regulates *FRE1* and *FRE7* through the Cu-metaloregulatory transcription factor, Mac1 (Martins et al. 1998). Consistent with its role in the reduction of Cu(II) and Fe(III) and its requirement for high affinity uptake of these metals, the *FRE1* gene is regulated by both Mac1 and Aft1 in response to Cu and Fe, respectively. Either subsequent to, or concomitant with reduction, Cu(I) is thought to be delivered to the plasma membrane associated, high affinity Cu transporters encoded by the *CTR1* and *CTR3* genes (Dancis et al. 1994a, Knight et al. 1996). It is currently unclear whether Cu(I) ion transfer occurs through a direct physical coupling between the FRE-encoded metalloreductases and Ctr1/3 at the plasma membrane, followed by Cu ion transport into the cytosol, or whether there may exist coupling molecules which serve as intermediate Cu carriers. High affinity uptake by Ctr1 and Ctr3 is specific for Cu and is saturable, with a K_m of 1–4 μmol/L as determined by ^{64}Cu uptake studies in whole yeast cells (Lin and Kosman 1990).

Although it was initially surprising that the Ctr1 high affinity Cu ion transporter was identified through a screen of mutants defective in Fe transport, this observation explains the long observed, but poorly understood link between the nutritional absorption of these two metal ions (see below). Indeed, chromosomal disruption of the *S. cerevisiae CTR1* gene results in several phenotypes associated with the lack of high affinity Cu uptake (Dancis et al. 1994a and 1994b). These include poor growth in low Cu media, respiratory deficiency (due to inability to provide Cu to cytochrome oxidase), sensitivity to oxidative stress (due to lack of Cu incorporation into Cu, Zn superoxide dismutase), inability to activate metallothionein expression (due to lack of Cu to activate the Cu-metalloregulatory transcription factor Ace1) and the absence of high affinity Fe transport (due to inability to provide Cu to Fet3, a multicopper ferroxidase required for high affinity iron uptake). Ctr1 is a 406 amino acid protein with three potential transmembrane domains. The amino terminal domain, which can be modeled based on membrane topological analysis to reside in the periplasmic space, contains several repeats of the putative metal binding motifs M-X-M or M-X_2-M (where X is any amino acid). In addition, the carboxyl terminal domain has cysteine, tyrosine and phenylalanine residues, all of which are potential metal binding ligands. The importance of these residues in Cu transport has yet to be determined. Ctr1 is highly glycosylated and forms oligomers at the plasma membrane (Dancis et al. 1994b). Interestingly, levels of Cu that exceed the K_m of Ctr1 trigger the rapid and specific degradation of Ctr1 at the plasma membrane (Ooi et al. 1996). This represents a novel mechanism for plasma membrane protein degradation. Studies using mutant yeast strains that have defects in the endocytic pathway and vacuolar degradation suggest that this process does not require internalization of Ctr1 or its delivery to the vacuole, where proteolytic degradation often occurs. However, at rather high Cu concentrations (10 μmol/L) it has been reported that Ctr1 also undergoes Cu stimulated endocytosis, which may play a role in Cu uptake or in further reducing the levels of Ctr1 at the plasma membrane under potentially toxic Cu ion concentrations.

A second high affinity yeast Cu transporter, Ctr3, was identified as a suppressor of the Cu starvation phenotypes associated with a *ctr1*Δ strain (Knight et al. 1996). Interestingly, *CTR3* gene expression in many laboratory strains of *S. cerevisiae* is extinguished by the insertion of a Ty2 retroviral-type transposable element, between the TATA box (binding site for the transcription initiation factor, TBP) and start site for *CTR3* transcription. Although it is conceivable that *S. cerevisiae* strains are at a disadvantage under some conditions when both Cu ion transporters are functional, a more likely explanation for its silence in some yeast strains is simply that many domesticated *S. cerevisiae* strains are derived from a common progenitor in which *CTR3* was randomly silenced by a chance transposition event (Knight et al. 1996). This is supported by the observation that while Ctr1 and Ctr3 can function independently to provide high affinity Cu transport to yeast, cells which possess both transporters have a distinct growth advantage under Cu-limiting conditions. Ctr3 is a 241 amino acid protein that has three putative transmembrane domains and 11 cysteine residues of which three pairs are arranged in a potential C-C or C-X_2-C metal binding motif. What remains curious is that, although Ctr1 and Ctr3 proteins function interchangeably in high affinity Cu transport, there is extraordinarily little sequence homology between these two proteins. Currently, there is no information to support the notion that Ctr1 and Ctr3 have distinct, yet partially overlapping roles, however, further studies are warranted to address this question. Recent studies indicate that Cu ion uptake in *S. cerevisiae* can also occur through at least three putative low affinity systems, encoded by the *FET4* (Dix et al. 1994 and 1997), *SMF1/2* (Liu et al. 1997, Liu and Culotta 1999) and *CTR2* genes (Kampfenkel et al. 1995). The physiological conditions under which these putative low affinity systems operate have not yet been defined.

It is interesting that thus far, none of the eukaryotic high affinity Cu ion uptake proteins thought to act at the plasma membrane have the hallmarks of ATPase domains. Although it is unclear what provides the energy to drive Cu ions across the plasma membrane, it is possible that the subunits that confer high affinity Cu ion transport are present in a higher order complex that couples to an ATPase. Alternatively, the potential role of potassium (or another ion) anti-port may serve as the driving force, however this must be reconciled

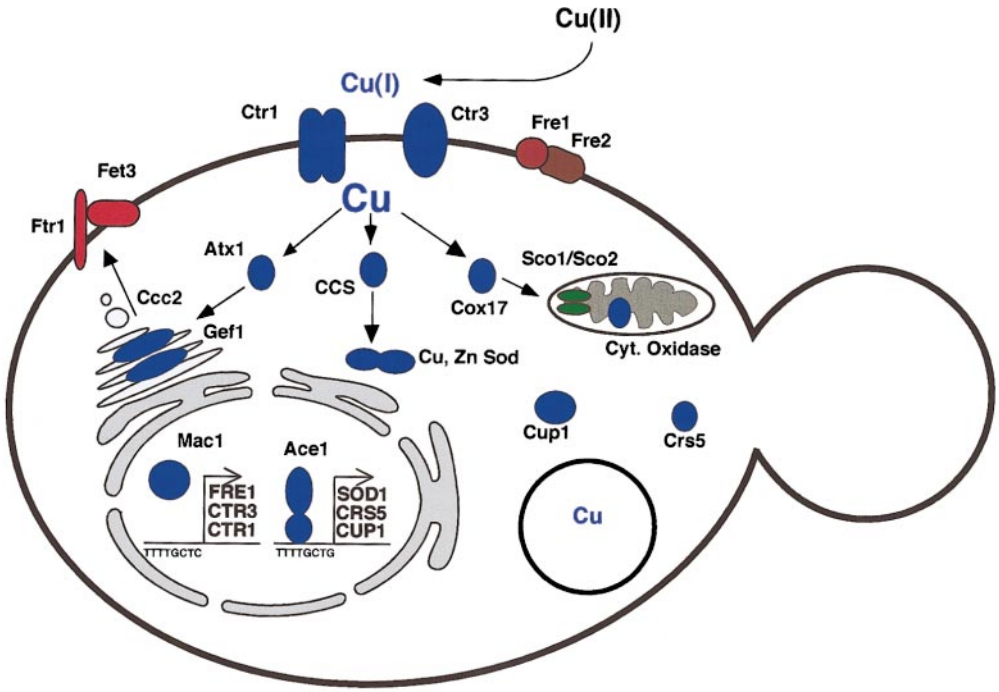
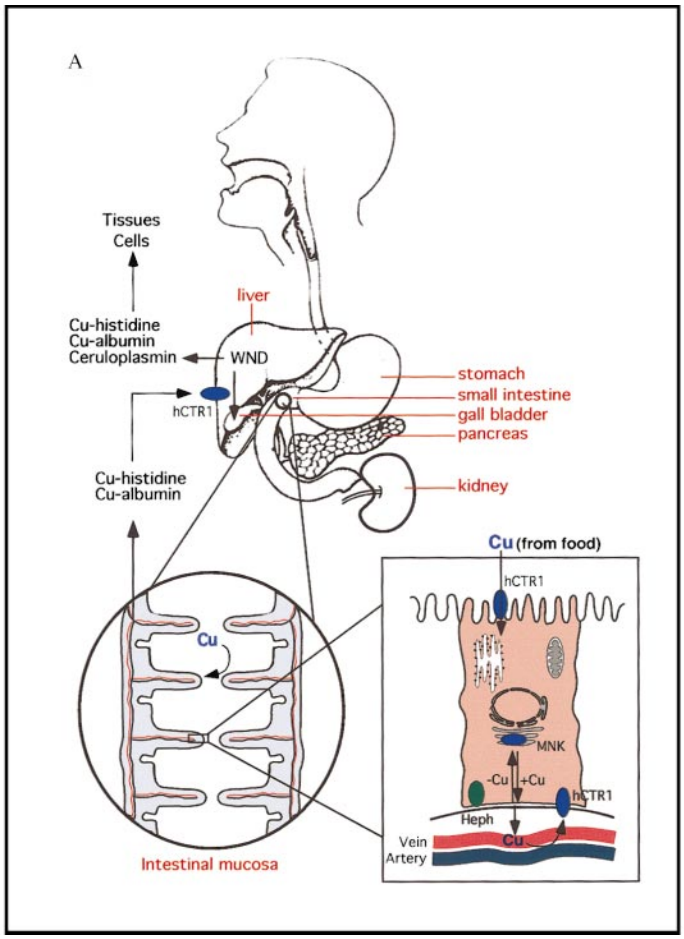
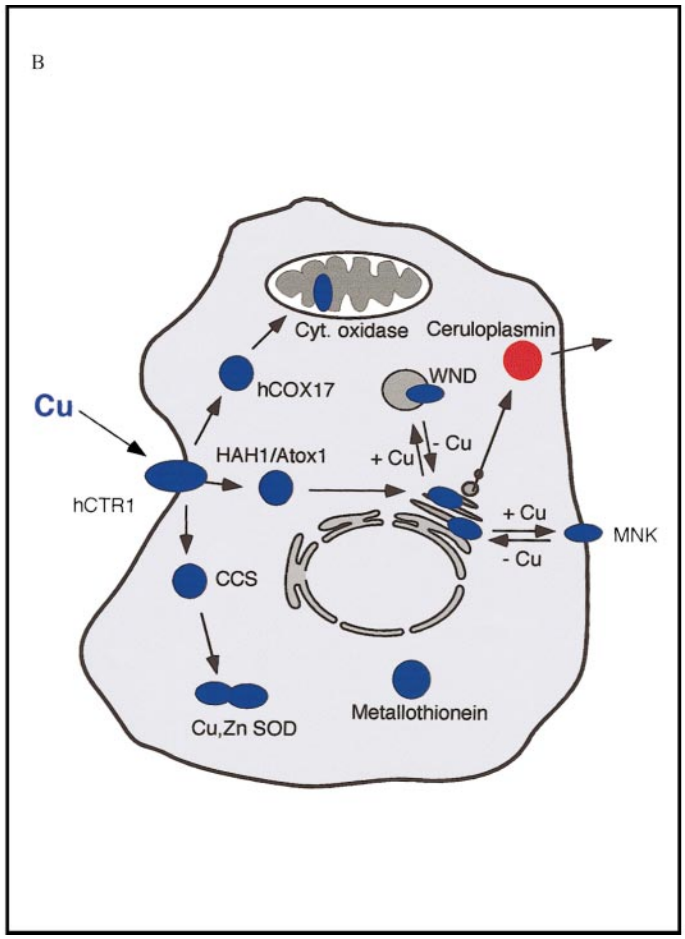


FIGURE 1 Copper transport and distribution in *Saccharomyces cerevisiae.* Copper is first reduced from Cu[II] to Cu[I] by cell surface reductases Fre1/Fre2 prior to uptake. High affinity Cu ion uptake is mediated by the Ctr1 and Ctr3 proteins. Within the cell Cu is bound to the known cytosolic Cu chaperones Atx1, Cox17 and CCS for specific delivery to the secretory pathway, mitochondria and Cu, Zn SOD, respectively. Within the secretory pathway, Ccc2 accepts Cu from Atx1, followed by incorporation of Cu to the multicopper ferroxidase, Fet3 in a manner facilitated by the Gef1 chloride channel. Fet3 forms a complex with the iron permease Ftr1 and both proteins are responsible for high affinity iron uptake at the plasma membrane. In mitochondria Cu delivered by Cox17 is incorporated into cytochrome oxidase, a process that requires the integral inner-membrane protein Sco1 and possibly its homologue, Sco2. CCS delivers Cu specifically to Cu, Zn SOD in the cytosol. Currently it is not known whether specific chaperones are required for incorporation of Cu to the metallothioneins Cup1 and Crs5 in the cytosol, or the nuclear Cu-metalloregulatory transcription factors Ace1 and Mac1 or the vacuole, which is important for proper Cu detoxification.





with the apparent ATP-dependence for high affinity Cu ion transport reported (Lin and Kosman 1990).

Absorption of Cu in mammals. The complexity of Cu uptake in mammalian systems is further compounded by the need to absorb Cu from the diet and distribute it throughout the tissues in the body. **Figure 2**A and B describe the overall scheme, as it is currently understood, for Cu absorption and distribution in humans, both at the organ and cellular level. What are the major questions that must be answered with respect to Cu absorption in mammalian systems? First, how is Cu extracted from nutrients and transported into mucosal cells that line the intestinal wall? Secondly, how does the absorbed Cu make its way from the intestinal mucosal cells through the baso-lateral membrane into the blood stream? Third, what are the ligands for this Cu in the bloodstream and how do they mobilize Cu throughout the body? Furthermore, how is this mobilized Cu re-transported into cells that comprise the body's organs?

In mammals, dietary Cu is absorbed across the mucosal membrane in cells which line the stomach and the small intestine (Crampton et al. 1965, Van Campen 1965). Exactly how this occurs is currently unknown. It has been proposed that Cu diffuses through the mucous layer that covers the intestinal wall (Linder 1991), however, given the exquisite care with which Cu is guided into and within yeast cells, it seems unlikely that diffusion alone would completely account for this process. Obviously, this is an important area that remains to be resolved in our understanding of Cu uptake in mammals, however, one candidate protein has been identified. A human complementary DNA (cDNA) encoding a putative high affinity Cu transport protein, denoted hCtr1, has been identified by functional complementation of the respiratory defect in yeast cells defective in Cu transport due to inactivation of both the *CTR1* and *CTR3* genes (Zhou and Gitschier 1997). hCtr1 is a 190 amino acid protein with significant homology to yeast Ctr1 and Ctr3, suggesting that mammalian high affinity Cu transporters may have evolved from both Ctr1 and Ctr3. The amino terminal domain of hCtr1 is rich in methionine and histidine residues, reminiscent of the methionine-rich amino terminal domain of yeast Ctr1. Furthermore, like the *S. cerevisiae* Ctr1 and Ctr3 proteins, topological analysis predicts the presence of three transmembrane domains. RNA blotting analysis has demonstrated that hCtr1 is expressed in all organs and tissues examined, with liver, heart and pancreas exhibiting the highest levels of expression, intermediate levels of expression in intestine while expression in the brain and muscle is low. Two major mRNA transcripts of 2 and 5.5 kb were detected with approximately the same ratios in all tissues. A less abundant 8.5 kb transcript was also detected. The presence of these different transcripts suggests the possible existence of forms of hCtr1 derived from alternative splicing events, perhaps with distinct functions in Cu transport. Whether hCtr1 plays an important role in Cu uptake into intestinal mucosal cells has yet to be firmly established.

A putative low affinity mammalian Cu transporter, hCtr2, was also identified by sequence homology with hCtr1 and the yeast Ctr1 and Ctr2 proteins (Dancis et al. 1994a, Kampfenkel et al. 1995, Zhou and Gitschier 1997). Like hCtr1, hCtr2 mRNA is detected in all organs and tissues examined, however, in this case the highest levels were observed in the placenta with very low abundance observed in the liver, ovary, intestine and colon. Unlike hCtr1, hCtr2 is unable to complement the respiratory defect in yeast strains defective in Cu transport, thus the role of Ctr2 in Cu homeostasis is unclear. A second putative low affinity Cu transporter, the Nramp2 protein, has also recently been identified as a proton-coupled metal ion transporter that transports a broad range of metal ions (Gunshin et al. 1997). It is interesting that Nramp2 protein is homologous to the Smf1/Smf2 yeast metal ion transport proteins (Liu et al. 1997, Liu and Culotta 1999).

Once transported into intestinal mucosal cells, how does Cu make its way through the baso-lateral membrane? Through the combined efforts of clinicians, molecular biologists and geneticists studying Menkes disease, an important component in this pathway has been identified. Menkes disease is characterized by progressive neurological impairment and death in infancy (Danks et al. 1973). The entrapment of Cu in intestinal cells, kidney and vascular endothelial cells in the blood-brain barrier in Menkes patients leads to Cu deficiency as ascertained by defects in the activities of many Cu-dependent proteins (Danks 1989). The isolation of the Menkes' disease gene (ATP7A) showed that this gene encodes a membrane-associated P-type ATPase (Chelly et al. 1993, Mercer et al. 1993, Vulpe et al. 1993). Like the *E. hirae* CopA protein, the Menkes protein (MNK) contains six successive repeats of the Cu-binding motif GMTCXXC within the amino terminal region. ATP7A mRNA is strongly expressed in the muscle, kidney, lung, and brain, but only a trace amount, if any, in the liver. Consistent with clinical data demonstrating accumulation of Cu in intestinal mucosal cells in Menkes patients, and a possible role for MNK in transport of Cu across the baso-lateral membrane, the ATP7A gene is expressed in intestinal epithelial cells (Murata et al. 1997).

Since studies to date indicate that the MNK protein is involved in both providing Cu to secreted Cu-metalloproteins, and Cu efflux from intestinal epithelial cells, where is the MNK protein localized? Studies in Cu-resistant Chinese ham-

FIGURE 2 Copper metabolism in humans. (*A*) Model for human Cu absorption and distribution at the organ and tissue level. Dietary Cu is primarily absorbed from the stomach and small intestine. hCTR1, a putative high affinity Cu transporter, may transport Cu into intestinal mucosal cells and MNK is required for Cu transport into the portal circulation. MNK is a P-type ATPase defective in Menkes patients in which Cu is accumulated in intestinal epithelial cells. Once entering the plasma, Cu is bound with albumin and histidine in the portal blood and rapidly deposited in the liver where hCTR1 may play a role in this process. Ceruloplasmin, a major Cu-containing protein in plasma, is synthesized in the liver with the incorporation of Cu by the WND protein in the secretory pathway and has ferroxidase activity that is critical for iron metabolism. WND has high homology with MNK and is defective in Wilson's disease patients who suffer from Cu accumulation in liver. Biliary excretion via the gall bladder is the major route of Cu elimination from the body and a small amount of Cu is found in urine. Heph is a membrane bound ceruloplasmin/Fet3 homologue required for iron egress from the intestine that is defective in *sla* mice, a model for sex-linked anemia. (*B*) Model for human Cu uptake and distribution at the cellular level. Tissue uptake of Cu is likely mediated by the hCTR1 Cu transporter. Once transported by hCTR1, the small cytoplasmic Cu chaperones (hCOX17, HAH1, CCS) distribute Cu to specific cellular compartments for the incorporation of Cu into Cu-requiring proteins. hCOX17, HAH1 and CCS deliver Cu to the mitochondria, secretory compartment, and Cu, Zn SOD, respectively. Cu chaperones for metallothionein and the nucleus have not been identified. In tissues other than the liver, MNK transports Cu delivered by HAH1 into the TGN for incorporation of Cu into secretory proteins. Although it is not known how these Cu chaperones take Cu transported from outside, the Atx1 and CCS Cu chaperones directly interact with their specific target molecule to surrender Cu. Cu stimulates the trafficking of MNK from the TGN to the plasma membrane where it may be involved in Cu efflux. In hepatic cells, WND, the Wilson's disease protein is localized in the TGN. Elevated Cu levels stimulates its trafficking from the TGN to an unknown cytosolic vesicular compartment.

ster ovary cells, in which the MNK genes have been amplified, showed that localization of MNK protein is regulated by Cu ion concentrations (Petris et al. 1996). In the presence of low Cu ion concentrations, the MNK protein is localized in the *trans*-golgi network (TGN) where it functions to deliver Cu to the secretory pathway. At elevated concentrations, Cu stimulates the trafficking of the MNK protein from the TGN to the plasma membrane where it is thought to be involved in Cu efflux to protect the cells from potentially toxic levels of Cu. MNK trafficking is energy-dependent, reversible and does not require the synthesis of new proteins. During this process, MNK protein levels and mRNA transcripts remain constant. The observation that Ag(I), which is chemically similar to Cu(I), also stimulates the translocation from the TGN to the plasma membrane suggests that it is the reduced form of Cu [Cu(I)], that activates this process in mammalian cells. Copper-dependent re-localization of the MNK protein requires at least two of the six metal binding motifs in its amino terminal domain (Strausak et al. 1999). According to recent reports, the third transmembrane region of MNK functions as a TGN targeting signal (Francis et al. 1998) and a carboxyl terminal di-leucine is required for recycling from the plasma membrane back to the TGN (Petris et al. 1998). Therefore consistent with a role in providing Cu to the secretory machinery, and in efflux of Cu into the circulation, the MNK protein moves to appropriate subcellular locations as a function of Cu ion concentration to carry out these duties.

Once Cu is pumped into the circulation, what are the recipient ligands and how do they mobilize Cu throughout the body? In the portal blood and systemic circulation Cu is bound to albumin and histidine (Linder 1991). Exactly how albumin and histidine relinquish Cu to organs and tissues is currently unclear, however, it is possible that the bound Cu is handed off to the hCtr1 Cu transporter, either directly or indirectly. The predominant Cu containing protein in mammalian serum is ceruloplasmin, a glycosylated multi-Cu ferroxidase synthesized primarily in the liver which carries $>$ 95% of total serum Cu (Holmberg and Laurell 1948). Ceruloplasmin coordinates seven Cu atoms that are incorporated during its biosynthesis and maturation in the secretory pathway (Sato and Gitlin 1991). Although it is not yet established whether ceruloplasmin is involved in Cu mobilization from the serum, the absence of ceruloplasmin in patients with aceruloplasminemia, a genetic disorder of ceruloplasmin deficiency, has not been shown to alter Cu levels in the peripheral tissues examined (Miyajima et al. 1987, Harris et al. 1998).

Normal physiological levels of Cu in mammals is maintained through a balance between absorption and distribution, and biliary and urinary excretion. A central location in the body for Cu metabolism is the liver. The importance of maintaining mechanisms for proper Cu homeostasis in the liver is underscored by the existence of the autosomal recessive disorder Wilson's disease (WND). Indeed, much like the study of mutants in bacterial or yeast cells informs us about fundamental biochemical mechanisms, the study of disease states in humans and animal models has been very informative with respect to Cu homeostasis. The ATP7B gene, which encodes the 160-kD WND P-type ATPase (Bull et al. 1993, Tanzi et al. 1993, Yamaguchi et al. 1993), is required for biliary excretion of Cu and incorporation of Cu into ceruloplasmin in the liver. Patients with Wilson's disease accumulate Cu in the liver and brain, resulting in liver cirrhosis, neurodegeneration and the formation of apo-ceruloplasmin. Indeed, recent gene therapy studies have demonstrated that when the ATP7B cDNA is introduced to the Long-Evans Cinnamon rat, a rodent model for Wilson's disease, through adenovirus mediated gene transfer, the synthesis of holoceruloplasmin is restored (Terada et al. 1998). Consistent with these observations, WND protein is localized to the *trans*-Golgi network in the liver and brain where it likely functions to incorporate Cu into ceruloplasmin (Nagano et al. 1998) and perhaps at the canalicular site plasma membrane of hepatocytes for excretion of Cu into the bile (Dijkstra et al. 1996). In the presence of elevated Cu concentrations, studies in HepG2 cells have shown that the WND protein moves from the TGN to a cytoplasmic vesicular compartment (Hung et al. 1997). Although the nature of this compartment is not well defined, perhaps the WND protein is en route to the bile canalicular membrane.

Interestingly, a 140 kD polypeptide representing a cleaved form of the WND protein was reported to be localized to the mitochondria in cultured hepatic cells and human tissues, rather than the TGN (Lutsenko and Cooper 1998). This form of WND protein is postulated to be a product of proteolytic cleavage within the metal binding repeats at the amino terminal domain and is targeted to the mitochondria where it is suggested to play a role in mitochondrial Cu ion homeostasis. How the alternative form of WND may play a role in mitochondrial function is currently unknown. To add to the complexity, an alternatively spliced form of WND, encoding a pineal gland night-specific ATPase (PINA), was found to be expressed in the pinealocytes and a subset of photoreceptors in adult rats, and transiently in the retinal pigment epithelium and ciliary body during retinal development (Borjigin et al. 1999). This novel splice variant of WND completely lacks the metal binding repeats and the first four putative *trans*-membrane segments of WND. Despite these deletions, PINA still modestly complements the defect of Cu incorporation into Fet3 associated with yeast *ccc2*Δ strains (see below), and is proposed to function as a Cu transporter in rat pinealocytes. Interestingly, PINA is expressed at a 100-fold higher level at night as compared to daytime. The discovery of PINA suggests a potential role for rhythmic Cu metabolism in pineal and/or retina circadian function and perhaps for other body tissues. It is intriguing that earlier studies have implicated Cu in circadian rhythms, suggesting that serum Cu levels vary as a function of day and night. Whether Cu regulates circadian rhythm, or circadian rhythm regulates Cu levels have not been clearly established. Transgenic mice, which harbor a complete deletion of PrP^{C}, the protein responsible for prion diseases, (see below) have only 20% of the normal Cu content in crude membranes, synaptosomes and endosomes taken from brain extracts (Brown et al. 1997). These mice exhibit alterations in their circadian rhythm and sleep patterns (Tobler et al. 1996).

Interestingly, the prion protein, PrP^{C}, whose post-translationally modified form Prp^{Sc}, is the causative agent in Creutzfeld-Jacob disease (CJD), kuru, Gerstmann-Straussler-Scheinker (GSS) disease and fatal familial insomnia (FFI), collectively known as prion diseases (Prusiner 1991), may play a role in Cu absorption in the central nervous system. PrP^{C} is a GPI-anchored cell-surface glycoprotein found in the brain, spinal chord and peripheral tissues. The normal physiological function of the prion protein is unknown, but the observations that it binds Cu(II) (Hornshaw et al. 1995a, Hornshaw et al. 1995b, Prince and Gunson 1998, Viles et al. 1999) and that it constantly recycles between the plasma membrane and an early endosomal compartment (Shyng et al. 1993 and 1994), suggests that it may be involved Cu uptake through some as yet unidentified mechanism. Recently, it was shown that Cu stimulates endocytosis of the prion protein (Pauly and Harris 1998). Like MNK protein movement, copper stimulation of PrP^{c} endocytosis requires the highly conserved Cu(II)-binding

repeats in the protein. It was also shown that Cu ions facilitate the renaturation of guanidine-denatured PrP^{Sc} molecules to form the protease-resistant infectious prion particle (McKenzie et al. 1998) that accumulates in the endosome due to defective recycling of this particle to the plasma membrane. Taken together, these observations suggest that while the normal physiological function of the prion protein may involve Cu uptake, Cu may play a role in the formation of the disease-causing particle by enhancing its endocytosis. The copper-dependent relocalization of the MNK, WND and PrP^{c} suggests that Cu binding can stimulate conformational changes in these proteins that are recognized by components of the endocytic pathway resulting in their movement between two different subcellular compartments.

Intracellular distribution of copper

Because of its highly reactive nature, it would be extremely harmful for Cu(I) to exist as a free ion in cells where it can participate in reactions whose products ultimately damage cell membranes, proteins and nucleic acids. Accordingly, experiments over the past few years have shown that upon transport into the cell, Cu is delivered to specific molecules or subcellular compartments through highly controlled pathways by forming complexes with small cytosolic proteins known as Cu chaperones. Atx1, a Cu chaperone that delivers Cu to the secretory pathway, was first identified as a suppressor of oxidative damage in yeast cells lacking superoxide dismutase (*SOD1*) (Lin and Culotta 1995, Lin et al. 1997). Atx1 is a 73-amino acid cytosolic polypeptide that contains a single amino terminal copy of the MTCXXC metal binding motif that is also found in other metal binding proteins (Lin and Culotta 1995). Extended X-ray absorption fine structure (EXAFS) measurements indicate that Atx1 indeed binds a single Cu(I) atom per polypeptide, as a two or three-coordinate complex with the thiol ligands of the two conserved cysteine residues and perhaps a third unidentified ligand (Pufahl et al. 1997). Upon binding Cu, the Atx1-Cu complex is proposed to make its way to the TGN where it delivers Cu by associating with an Atx1-like amino terminal domain in Ccc2 protein through a series of two or three-coordinate Cu-bridged intermediates (Pufahl et al. 1997, Ralle et al. 1998). The yeast Ccc2 protein is a TGN localized ortholog of the mammalian MNK and WND P-type ATPases that transports Cu into the secretory compartment (Yuan et al. 1995). A number of experimental results support the model that Atx1 delivers Cu to Ccc2 in the TGN, followed by incorporation of Cu into Fet3, the multicopper oxidase that is an essential component of the high affinity Fe transport complex (Lin et al. 1997, Pufahl et al. 1997). Chromosomal disruption of the *ATX1* gene renders cells unable to grow in low Fe medium presumably due to reduced high affinity Fe uptake resulting from lack of Cu incorporation into Fet3. This defect can be corrected by addition of Cu to the growth medium, suggesting that delivery of Cu into Fet3 is impaired in these mutants. In vivo labeling experiments with ^{64}Cu confirmed that indeed, incorporation of Cu to Fet3 is defective in these mutants. The poor growth of *atx1*Δ mutants in low iron media can also be corrected by over-expression of *CCC2*, consistent with the model that Atx1 delivers Cu ions to Ccc2 prior to incorporation into Fet3. Furthermore, protein-protein interaction (two-hybrid) experiments have shown that Atx1 interacts specifically in a Cu-dependent manner with the amino terminal metal binding domain of Ccc2 (Pufahl et al. 1997). Consistent with this interaction and the delivery of Cu to Ccc2 by Atx1, a metal binding repeat in the MNK protein has been demonstrated to bind Cu(I) via two cysteine residues (Ralle et al. 1998). Recent studies have shown that in addition to Ccc2, efficient delivery of Cu to Fet3 requires Gef1, a homologue of a mammalian CLC chloride channel that is found in the *trans*-golgi network (Davis-Kaplan et al. 1998, Gaxiola et al. 1998). Gef1 is believed to act as an anion channel to provide a counterbalancing charge that will permit the delivery of Cu cations into a subcellular compartment that is highly acidic.

The discovery that Cu must be supplied to an essential component of the yeast high affinity Fe transport complex explains the old observation of an inextricable link between Cu and Fe uptake. Early studies showed that Cu-deficient swine developed microcytic hypochromic anemia which can be corrected by supplementing their diet with Cu but not with Fe (Lee et al. 1968). Cu supplementation lowered the abnormal Fe accumulation in these swine and increased Fe absorption into body tissues, strongly suggesting that Cu is essential for efficient iron uptake and mobilization in mammals. Similarly, in the absence of functional yeast high affinity Cu uptake systems, Fet3 exists as an inactive apoprotein, and as a consequence, the Ftr1 Fe permease protein fails to assemble properly at the plasma membrane (Stearman et al. 1996). As a consequence these cells are starved for Fe. This model to explain the strict requirement for Cu in Fe uptake and mobilization appears to be conserved in mammals at least in terms of the fundamental proteins involved in this process. Recent studies of mice with sex-linked anemia (sla) that accumulate Fe in intestinal enterocytes have been shown to be defective in a gene, *Heph*, encoding an integral membrane ceruloplasmin homologue (Vulpe et al. 1999). The Heph protein is thought to be essential for Fe egress from the intestinal enterocyte baso-lateral membrane into the portal circulation, providing an explanation for the observed anemia in these animals. While Fet3 and Heph are thought to transport Fe in opposite directions, the similarity in sequence and Cu-dependence between these proteins is striking.

It is clear that there is a complex network of factors involved in the distribution of Cu to distinct subcellular compartments and proteins. Another Cu chaperone discovered in yeast, Cox17, has been shown to play an essential role in the delivery of Cu to the mitochondria for respiratory function (Glerum et al. 1996a). Cox17 is a 69-amino acid polypeptide which partitions between the cytosol (40%) and the intermembrane space of the mitochondria (60%) (Beers et al. 1997). Cox17 contains seven potential metal binding cysteine residues and EXAFS analysis of metallated Cox17 has demonstrated that it binds two Cu(I) ions, within a labile binuclear cluster, via trigonally coordinated thiolate ligands (Srinivasan et al. 1998). Consistent with the function of Cox17 to deliver Cu to mitochondria, inactivation of the *COX17* gene results in loss of cytochrome oxidase activity due to a failure in the assembly of a functional multi-subunit complex leading to a corresponding respiratory deficiency. This defect can be suppressed by the addition of high levels of Cu to the media or by over-expression of the *SCO1* gene, or to a lesser degree the *SCO2* gene, encoding integral membrane proteins located in the inner membrane of the mitochondria (Glerum et al. 1996b). The generation of yeast *sco1*Δ mutants has demonstrated that the Sco1 protein is required for the assembly of cytochrome oxidase. Furthermore, the observation that Sco1 harbors a potential Cu-binding domain much like that observed in cytochrome oxidase suggests a model in which Cox17 may hand off Cu to the Sco1 receptor, which transfers the metal ion into the cytochrome oxidase active site. The observation that *cox17*Δ mutants are not defective in Cu, Zn superoxide dismutase activity, and over-expression of Atx1 in

*cox17*Δ mutants does not correct the corresponding defects, suggests that Cox17 activity is largely specific for the mitochondria.

The Atx1 and Cox17 proteins specifically deliver Cu to intracellular compartments, the secretory pathway and mitochondria, respectively. Insight into how cytosolic proteins acquire Cu came with the discovery of yeast CCS (the Copper Chaperone for SOD1), a 249-amino acid protein required for the direct incorporation of Cu into Cu, Zn SOD (Culotta et al. 1997). Cells that harbor a chromosomal deletion of *CCS* have normal levels of Cu, Zn SOD protein but the enzyme is inactive due to failure to incorporate Cu. These cells have no defect in Fe uptake or cytochrome oxidase activity or the ability to activate *CUP1* expression via the Ace1 transcription factor, suggesting that the defect is specific to SOD1 Cu loading. The mechanism of Cu transfer from CCS to Cu, Zn SOD has not been characterized, however, CCS appears to have two domains; an Atx1-like domain which harbors the metal binding motif, MTCXXC, and a second domain which is similar in sequence to a region of Cu, Zn SOD. Delivery of Cu to Cu, Zn SOD could, in principle, occur in two steps. First, CCS could coordinate Cu through the conserved MTCXXC motif in the Atx1-like domain. Secondly, a protein-protein interaction between the CCS SOD1-like domain and the Cu, Zn SOD monomer could facilitate metal transfer from the metal-binding site in CCS to the copper binding site in SOD. Perhaps the SOD-like domain of CCS competes with SOD in homodimer formation sufficiently long to allow transfer of Cu into the active site of the monomer, followed by homodimerization of the metallated subunits to give Cu, Zn SOD.

Given the strong conservation of structure and function between yeast and humans, it is not surprising that the intracellular distribution of Cu ions through Cu chaperones that is observed in yeast, appears to be conserved in mammalian cells. A human Atx1 homolog (Hah1) (Klomp et al. 1997) has been identified; it shares structural similarity to Atx1, and it complements the defects associated with a yeast *ATX1* gene deletion. Like Atx1, the human Hah1 (Atox1) may function to bind Cu and supply it to the Wilson's or Menkes disease proteins in the TGN. The other yeast Cu chaperones, CCS and Cox17, also have human homologs. The human homolog of yeast CCS, hCCS, directly interacts with cytoplasmic superoxide dismutase (SOD1) (Casareno et al. 1998). The relative cellular distribution of hCCS in brain appears to parallel that of SOD1, consistent with a biochemical relationship between these proteins (Rothstein et al. 1999).

A requisite mode for metal binding by the Cu chaperones must be that the Cu ions are coordinated to maintain a stable complex as it approaches its target molecule or compartment, and yet the Cu must be kinetically labile to allow metal ion exchange to take place for transport and distribution. A number of important questions remain to be solved with respect to Cu chaperone structure, function and mechanism of action. The observed specificity of each chaperone for a particular target suggests the existence of additional Cu chaperones. For example, it is possible that a chaperone exists for targeting Cu into the yeast nucleus for incorporation into the Cu metalloregulatory transcription factors Ace1 and Mac1. In addition, the vacuolar H^+-ATPase encoded by *VMA3,* as well as the *PEP3* and *PEP5* gene products required for vacuolar assembly, are also required for appropriate Cu ion detoxification (Eide et al. 1993, Szczypka et al. 1997). It has been proposed that excess Cu is stored in the vacuole to prevent toxicity. The role of putative Cu chaperones for targeting Cu to the vacuole and their mechanism of action is unknown. Furthermore, the mechanism by which Cu ions are delivered from the plasma membrane transporters to the Cu chaperones without releasing free Cu ions into the cytosol remains unclear. It is possible that the transporters may directly interact with the chaperones or that there may be a central Cu ion receptor at which Cu ions are incorporated into the chaperone. The specificity of each chaperone for its target subcellular compartment or protein is intriguing. It is possible that there may be a competition among the chaperones for Cu ions and that a shift in intracellular conditions resulting from external stresses may affect the levels of Cu chaperone expression and thereby determine the direction of Cu ion trafficking. For example, expression of *ATX1* is partially regulated by the iron sensor, Aft1 (Lin et al. 1997). Under conditions of iron limitation, it is possible that increased expression of *ATX1* redirects Cu to the secretory pathway to enhance high affinity iron uptake. Transcriptional regulation of *COX17* and *CCS* by metal ions has not been reported, but it is possible that under conditions of oxidative stress, CCS protein could direct Cu ions to Cu, Zn SOD for detoxification of superoxide anions, or that during nonfermentative growth, delivery of Cu ions to the mitochondria for incorporation into cytochrome oxidase may be favored to allow respiration. In addition, it is not known if other Cu-containing enzymes such a lysyl oxidase, dopamine β-hydroxylase, or others, have their own Cu chaperones or if they share common chaperones which deliver Cu ions to the subcellular organelle where they are assembled or active. One might expect, for example, that all secreted proteins that utilize the classical secretory pathway may obtain their Cu through Atx1/Hah1. While several questions remain unanswered, the identification of the Cu chaperones has tremendously enhanced our understanding of the mechanisms by which Cu is partitioned within the cell.

SUMMARY AND PERSPECTIVES

The large repertoire of cellular proteins with a role in Cu ion homeostasis underscores the obvious complexity of managing an essential, yet highly toxic nutrient. The use of both prokaryotic and yeast microbial model systems, and their powerful genetic and molecular tools, has greatly facilitated our identification of the components involved in Cu uptake and distribution and their mechanisms of action. Many of these components are both structurally and functionally conserved in mammals. Although Cu-responsive transcription factors similar to those identified in yeast have not been described in mammals to date, perhaps the Cu-sensing domains used by these factors will be found among Cu-sensing and signaling proteins in mammalian cells. Many questions remain to be elucidated with respect to Cu transport and intracellular distribution. What are all of the proteins involved in this process? How is specificity achieved and how are cellular membranes protected from the potential deleterious effects of Cu as it traverses the membrane? What is the driving force that allows Cu ion transport to occur? How does the transport machinery surrender the Cu, once transported into cells, to specific receptors? How is Cu carefully mobilized and targeted within cells to appropriate recipient proteins and compartments? Understanding these and other fundamental aspects of Cu transport and distribution will aid in our understanding of how this essential nutrient is balanced in living systems. The underlying principles for the answers to many of these questions undeniably reside in the chemical properties of Cu ions and the specific interactions of Cu with proteins.

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