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Maintaining copper homeostasis: regulation of copper-trafficking proteins in response to copper deficiency or overload

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Abstract

Copper is an essential micronutrient that plays a vital role as a catalytic co-factor for a variety of metalloenzymes. The redox chemistry of copper also makes it a potentially toxic metal if not properly used. Therefore, elaborate mechanisms have evolved for controlling its cellular uptake, elimination, and distribution. In the last decade, our understanding of the systems involved in maintaining copper homeostasis has improved considerably with the characterization of copper transporters that mediate cellular copper uptake or efflux and with the identification of copper chaperones, a family of proteins required for delivering copper to specific targets in the cell. Despite the distinct roles of these proteins in copper trafficking, all seem able to respond to changes in copper status. Here, we describe recent advances in our knowledge of how copper-trafficking proteins respond to copper deficiency or overload in mammalian cells in order to maintain copper balance.

Keywords: Copper transporters; Copper chaperones; Deficiency; Overload; Regulation

1. Copper is an essential and toxic metal

Copper is an essential micronutrient required by all living organisms [1]. The relative ease by which copper can be converted between different redox states such as oxidized Cu(II) and reduced Cu(I) has been exploited by organisms, and has led to its use as a catalytic co-factor for a variety of metalloenzymes involved in many diverse biological processes. The reactive nature of ionic copper also makes it a toxic metal if not properly handled by the cell. In the cell, under normal conditions, free copper is virtually nonexistent as the cell has an overcapacity for copper sequestration [2]. However, under conditions of copper overload, free copper ions can accumulate and react to generate hydroxyl radicals that can engage in reactions that can adversely modify proteins, lipids, and nucleic acids [3]. Free copper ions may also exert their toxic property by displacing other essential metal co-factors from metalloenzymes. For example, it has been reported that copper can substitute for Zn(II) in zinc-finger transcription factors that renders the proteins unable to bind their target sequence [4]. Cellular copper concentrations must therefore be maintained at levels where nutritional deficiency and toxicity are avoided. From unicellular organisms to specialized cells of mammals, elaborate mechanisms have evolved to efficiently acquire and properly utilize copper.

In the last decade, our understanding of how cellular copper homeostasis is maintained at the molecular level has greatly improved with the characterization of copper transporters that mediate cellular copper uptake or egress and with the discovery of a novel family of proteins, termed "copper chaperones," that function to deliver copper to specific targets in the cell. Copper transporters and chaperones identified in lower eukaryotes are also present in mammals, indicating remarkable evolutionary conservation of the systems involved in copper trafficking within cells. This review will focus on recent advances in our understanding of the regulation of copper-trafficking proteins in response to copper deficiency or overload required for maintaining cellular copper homeostasis in mammals.

2. Copper requirements

Copper plays a vital role as a co-factor for a number of metalloenzymes including Cu/Zn superoxide dismutase (an-

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tioxidant defense), cytochrome c oxidase (mitochondrial respiration), lysyl oxidase (development of connective tissue), tryrosinase (melanin biosynthesis), ceruloplasmin (iron homeostasis), hephaestin (intestinal iron efflux), dopamine β-hydroxylase (catecholamine production), and peptidylglycine α-amidating mono-oxygenase (peptide hormone processing) [5,6]. Many of the symptoms associated with copper deficiency are a consequence of decreased activity of copper-dependent enzymes [7–10]. However, other than in specialized situations such as with malabsorption syndromes, malnutrition, and genetic disorders of copper metabolism, overt copper deficiency is relatively rare in humans, indicating the presence of efficient mechanisms for absorbing copper from foods and distributing the metal to essential sites in the body. Once absorbed, copper is rapidly distributed to copper-requiring enzymes and only a small fraction is stored in the body. Regulation of total body copper occurs largely at the small intestine, the major site of copper absorption. The amount of copper absorbed from the diet varies considerably with dietary copper intake [11,12]. When intake is less than 1 mg/day, more than 50% of the copper is absorbed; in comparison, when copper intake is more than 5 mg/day, less than 20% is absorbed [12]. Total body copper levels are also controlled at the liver, which is the principal storage site for copper and regulates its excretion into the bile [11–13]. Dietary intakes of copper for adults range from 0.6 to 1.6 mg copper/day [14], most of which comes from eating foods rich in copper such as seafood, organ meats, nuts, and seeds [15]. The Recommended Dietary Allowance for adult men and women is 0.9 mg copper/day and the Tolerable Upper Intake Level has been set at 10 mg/day [14].

3. Menkes and Wilson copper transporters

Much of our understanding of the cellular trafficking of copper has come from studies conducted in prokaryotes and lower eukaryotes such as the yeast *Saccharomyces cerevisiae*. In contrast to *S. cerevisiae*, where regulation of many genes encoding for proteins involved in copper metabolism is mediated by copper-sensing transcription factors that modulate gene expression [16,17], it seems that mammalian cells maintain copper homeostasis mainly by controlling the activity of copper-trafficking proteins by post-translational mechanisms. Figure 1 summarizes the copper-trafficking pathways within a mammalian cell and regulation of copper-trafficking proteins in response to copper deficiency or overload as presently understood.

Disorders of copper metabolism were instrumental in forwarding our understanding of how copper is distributed in the body. Menkes syndrome and Wilson's disease, disorders of copper deficiency and overload, respectively, led to the discovery of the gene products mutated in these diseases. ATP7A (Menkes protein) and ATP7B (Wilson protein) are P-type ATPases that have eight transmembrane domains and contain six MXCXXC (M = methionine; C = cysteine; X = any other amino acid) copper-binding motifs at the N-terminus. P-type ATPases are a family of cation transporters that use energy from ATP hydrolysis to translocate metal cations across lipid bilayers. ATP7A transcript is expressed at high levels in muscle, kidney, lung, and brain but is expressed at low levels in placenta and pancreas, and only trace amounts are detected in the liver [18,19]. In contrast, ATP7B transcript is strongly expressed in the liver and kidney [20,21].

Under normal copper conditions, both ATP7A and ATP7B are predominately localized to the trans-Golgi network (TGN) [21,22], where they function in the copper secretory pathway, delivering copper for incorporation into nasent cuproproteins. This is evident in Wilson's disease, which results in a dramatic reduction in ceruloplasmin activity due to impaired copper incorporation into apoceruloplasmin [23]. When the cell is exposed to high levels of copper, ATP7A redistributes from the TGN to the cell surface where ATP7A is thought to rid the cell of excess copper in response to copper overload [24,25]. Ag(I), which is similar to Cu(I) in structure, also stimulates this translocation, suggesting that the reduced form of copper activates this process [24]. Mutational analysis of the Menkes protein has revealed an important role for the two metal-binding sites (MBS) close to the membrane channel for copper-dependent trafficking [26]. However, other studies have indicated that the MBS are not absolutely required for trafficking [27], and it has been postulated that the MBS may act as copper sensors [28].

ATP7B displays a similar copper-dependent translocation in response to increased cellular copper concentrations. ATP7B moves from the TGN to a cytoplasmic vesicular compartment concentrated near the hepatocyte canalicular membrane in response to elevated copper, where it is thought to function in copper excretion into the bile [29]. This vesicular localization may also reflect a role for the Wilson protein in sequestering copper into the lumen of vesicles, which may prevent excess copper from engaging in deleterious reactions in the cell. How copper is exported from the cell once inside these vesicles is not clear. The Wilson protein may traffic to the plasma membrane at low levels to expel copper [30], or the copper inside the vesicles may be exocytosed while the Wilson protein returns to the TGN [31–33]. The change in subcellular distribution of both transporters is reversible and independent of new protein synthesis, indicating efficient recycling of these transporters [22,24,29,31]. In addition, the expression level of neither transporter is altered by changes in cellular copper concentrations [24,29]. Of note, a 140-kD form of the Wilson protein was reported to be localized to the mitochondria rather than the TGN, and this variant is thought to function in maintaining mitochondrial copper homeostasis [34]. Furthermore, an alternatively spliced variant of the Wilson protein, pineal night-specific ATPase (PINA), has been detected in pinealocytes, a subset of photoreceptors, and in the

retinal pigment epithelium and ciliary body. Interestingly, PINA is dramatically up-regulated in the night-time compared with the daytime [35].

4. Ctr1 and Ctr2 copper transporters

S. cerevisiae have two transporters that mediate high affinity copper uptake (yCtr1 and yCtr3) and that appear to be functionally redundant, although they share little homology in primary sequence. Zhou et al. [36] isolated the human high-affinity copper transporter 1 (hCtr1), and it was determined to be 29% identical to Ctr1 of *S. cerevisiae*. Human Ctr1, although significantly smaller than its yeast orthologue (190 amino acids compared with 406), contains a similar methionine and serine rich N-terminus and can complement phenotypes of a yeast strain lacking the high-affinity copper uptake transporters [36]. Ctr1 mRNA is ubiquitously expressed, with the highest levels found in the liver and lower levels detected in the brain and spleen [36]. Northern blot analysis using human tissues revealed that hCtr1 has two major transcripts of approximately 2 and 5.5 kb in size and a less abundant transcript of 8.5 kb [36]. Ctr1 has three transmembrane domains, exists as a homotrimer, and has been shown to be extensively glycosylated [37–40].

Targeted deletion of the *Ctr1* gene in mice has revealed an important role for Ctr1 in embryonic development, as $Ctr1^{-/-}$mice die at approximately mid-gestation [41,42]. Heterozygous mice show a tissue-specific reduction in copper content, with copper levels in the brain and spleen being about half those of their wild-type littermates [41]. Cuproenzyme activity in $Ctr1^{+/-}$mice is also significantly decreased [41]. Interestingly, Ctr1 mRNA is expressed at low levels in the brain and spleen compared with the liver and kidney, where $Ctr1^{+/-}$ mice show no reduction in copper content compared with that of wild-type mice. These data may suggest the presence of Ctr1-independent copper uptake systems in certain tissues but not in others. Alternatively, Ctr1 expression in brain and spleen may just be sufficient to meet the copper requirements for these tissues and any reduction in Ctr1 activity would be reflected by a decrease in tissue copper content. However, if the latter were true, these results would be inconsistent with the presence of a post-translational regulation mechanism for inducing Ctr1 activity to increase copper uptake in response to copper deficiency in the brain and spleen. Elucidation of the mechanisms underlying the tissue-specific copper deficiency observed in Ctr1-deficient mice will be of significant interest.

Over-expression experiments in culture have revealed that uptake of copper by Ctr1 is energy independent and is stimulated by acidic extracellular pH and high K^+concentrations [37]. Also, uptake of copper by Ctr1 is time dependent and saturable [37]. The stimulation of copper uptake by K^+ and the observation in yeast that copper uptake is coupled with K^+ efflux may indicate that copper uptake by Ctr1 is mediated by a $Cu^+/2K^+$ antiport mechanism [43,37].

Ctr1 has been variously reported to be localized to a perinuclear compartment, cytoplasmic vesicles, and cell surface [37,38,44]. Ctr1 has been shown to localize at the cell membrane in copper-deficient cells; upon exposing cells to high copper concentrations, Ctr1 rapidly internalizes and displays a cytoplasmic vesicular distribution [44]. In HEK293 cells, upon blocking protein synthesis, Ctr1 degradation was shown to be more rapid when cells are cultured in medium containing high concentrations of copper [44]. The reported copper-dependent change in subcellular localization of Ctr1 may be a mechanism for controlling cellular copper influx. Distribution of Ctr1 to the cell surface under copper-deficient conditions may increase the rate of copper uptake by the cell and alternatively, internalization of Ctr1 when copper levels are high may reduce uptake. Of note, tissue-specific localization and discrepancies in the copper-dependent trafficking of Ctr1 in response to copper deficiency have been reported [37,38,44], findings that may reflect tissue-specific regulation of Ctr1 activity. Whether expression level of Ctr1 or a change in its subcellular localization in response to copper availability plays a significant role in regulating cellular copper concentrations awaits confirmation from other studies.

Based on sequence homology with Ctr1, the human gene for another putative copper transporter, copper transporter 2 (hCtr2), was identified [36]. Even though the topology and size of Ctr2 is similar to that of Ctr1, Ctr2 has a lower abundance of histidine and methionine residues and does not have well defined copper-binding motifs [36]. Moreover, the mRNA tissue expression patterns of Ctr1 and Ctr2 are significantly different, with Ctr2 being abundantly expressed in the placenta and lower levels detected in the liver [36]. This difference in tissue expression patterns and experiments in yeast, demonstrating that Ctr2 does not efficiently complement phenotypes of mutant yeast deficient in high affinity copper uptake [36,45], suggests distinct roles for Ctr1 and Ctr2 in copper trafficking. Recently Ctr2 has been localized to the yeast vacuole and not the cell surface [46], providing further evidence in support of an alternative role for Ctr2 in copper metabolism. It has been postulated that Ctr2 may function in the distribution or mobilization of intracellular copper pools [46]. Recent data from our laboratory has shown that Ctr2 expression is down-regulated in tissues of copper-deficient rats and is localized to a perinuclear compartment and cytoplasmic vesicles (unpublished results). Understanding the mechanisms underlying the regulation of Ctr2 in response to copper deficiency and ablation of Ctr2 expression in mice will provide important information as to the role of this protein in copper trafficking.

Interestingly, $Ctr1^{-/-}$-deficient cells still accumulate copper [47], indicating the presence of Ctr1-independent mechanisms for copper acquisition by cells. Given that Ctr2 cannot complement phenotypes of yeast deficient in high

affinity copper uptake, it would seem unlikely that Ctr2 would account for the copper uptake observed in Ctr1-deficient cells, although confirmation must await characterization of Ctr2 function in mammalian cells. The divalent metal transporter 1 (DMT1; also known as DCT1 or Nramp2), an iron transporter that has been shown to uptake in addition to iron other divalent metals including copper, may mediate copper uptake in Ctr1-deficient cells. Treatment of intestinal Caco-2 cells with a DMT1 antisense oligonucleotide has been shown to reduce copper uptake, indicating that DMT1 may be important for copper absorption at the intestinal level [48]. However, DMT1-deficient mice do not show copper deficiency [49], and copper uptake by $Ctr1^{-/-}$ embryonic cells is not competed by iron [47]. Research directed toward characterization of Ctr1-independent cellular copper uptake systems will be of great importance.

5. Murr1

Recently, Murr1 has been identified as the gene mutated in inbred Bedlington terriers that develop hepatic copper toxicosis [50]. Murr1 is a small cytoplasmic protein that is also found in human liver [50]. Although the function of Murr1 in copper metabolism is unknown, it has been proposed that it may play a role in vesicular copper movement and excretion at the canalicular membrane of hepatocytes [50]. Consistent with a role in copper excretion, Murr1 has recently been shown to directly interact with the Wilson protein [51]. Copper-dependent regulation of Murr1 has not yet been reported.

6. Copper chaperones

The discovery of copper chaperones revolutionized our thinking of how copper is routed within cells. In 1995, Lin and Culotta identified the yeast ATX1 (antioxidant protein 1) as a putative antioxidant protein that, when expressed in yeast, suppressed oxidative damage in cells deficient in Cu/Zn superoxide dismutase (SOD1) [52]. Later it was shown that ATX1 could transfer copper to the yeast homologue of the Menkes and Wilson transporters, Ccc2 [53]. ATX1 became the prototype member of a family of proteins termed copper chaperones that appear to have the sole function to deliver copper to specific cellular compartments and targets. The mammalian orthologue of ATX1, ATOX1 (also known as HAH1) [54] was identified and shown to transfer copper to the Menkes and Wilson transporters by direct protein–protein interaction that is stimulated by the presence of copper [55,56]. Recently, ATOX1 has been shown to play a critical role in modulating the copper-dependent movement of ATP7A from the TGN to the cell surface and to determine the threshold for copper-dependent trafficking of ATP7A [57]. Generation of ATOX1 null mice revealed an essential role for this copper chaperone in perinatal copper homeostasis, as these mice show elevated mortality after birth and display other defects including growth retardation, skin laxity, hypopigmentation, and seizures [58]. Consistent with a role for ATOX1 in copper delivery to the Menkes and Wilson proteins, ATOX1-deficient cells accumulate high levels of copper due to a defect in cellular copper efflux [57].

The abundant antioxidant enzyme, SOD1, obtains its copper co-factor through its copper chaperone, copper chaperone for SOD1 (CCS). We have reported that CCS is up-regulated in a dose-dependent manner in various tissues of rats fed low-copper diets [59]. We determined that CCS up-regulation is specific for copper deficiency, and that copper deficiency reduces the rate of CCS degradation by the 26 S proteosome [60]. Increased expression of CCS when copper is scarce likely increases the efficiency of copper transfer to SOD1, suggesting a role for CCS in prioritizing the utilization of cellular copper when it is limiting. In contrast to CCS, Hamza et al. have reported that the expression of the copper chaperone HAH1 is not affected by copper deficiency in HeLa cells [55]. Although it is not known where copper chaperones obtain copper in the cell, if both CCS and HAH1 acquire copper from the same cellular pool, under conditions of limiting copper it is likely that copper is preferentially directed toward incorporation into SOD1 as opposed to the Menkes and Wilson transporters. Copper chaperones may therefore play a role in determining the hierarchy of copper utilization in copper-deficient cells.

To date, several other copper chaperones have been identified including Cox17, which delivers copper to the mitochondria [61,62], and the mitochondrial proteins SCO1 and SCO2, which may function downstream of Cox17 to incorporate copper into cytochrome c oxidase (COX) [63,64]. Recently mutations in SCO1 and SCO2 in humans have been associated with diseases showing COX deficiency [65–67]. A variant transcript of the Menkes gene has also been identified in several human cell lines that encodes for a protein that is targeted to the nucleus [68]. Thus, this variant was referred to as *n*uclear *M*enkes-*l*ike 45 (NML45), and this protein may function as a copper chaperone that shuttles copper to the nucleus. Studies examining how the activities of copper chaperones and their respective enzymes are influenced by copper deficiency or overload will provide valuable insight into the distribution and utilization of cellular copper when it is abundant or scarce.

7. GSH and metallothioneins

The tripeptide glutathione (GSH) is present at high concentration in a number of tissues [69], and has been shown to bind Cu(I) and to play a role in biliary excretion of copper [70,71]. In addition, GSH can transfer copper to cuproproteins including metallothioneins [72–74], a family of pro-

teins that play an important role in metal detoxification. It has been known for some time that intake of high quantities of zinc increases the level of metallothioneins and reduces the absorption of copper [75,76]. It has been proposed that sequestration of copper by metallothioneins in mucosal cells may account for the decrease in copper absorption [75,77,78].

Liver cells of copper-deficient rats have increased amounts of GSH [79] and cells with reduced GSH levels have been shown to be slower in copper uptake from medium and to have a lower steady-state copper concentration [80]. Injection of copper into rats has been reported to decrease GSH levels in the liver [81]. Together, these studies support a role for GSH and metallothioneins in intracellular copper metabolism that extends beyond their role as metal detoxification proteins.

8. Prion protein

There is substantial data that suggest a link between copper and the prion protein (PrP^{C}). A mutant form of the protein (PrP^{Sc}) is the culprit in a number of neurodegenerative disorders. PrP^{C} is a glycosylphosphatidylinositol-anchored protein that is expressed in the central nervous system as well as peripheral tissues. Thus far, no known function has been found for PrP^{C}. It has been shown that copper can bind the PrP^{C} *in vivo* [82] and can rapidly stimulate endocytosis of PrP^{C} from the cell surface in a process that is reversible [83]. Furthermore, copper binding at the outer plasma membrane has been shown to be related to the expression level of PrP^{C} [84]. Whether PrP^{C} plays a significant role in the uptake or intracellular trafficking of copper remains to be determined.

9. Conclusion

Despite the diversity of proteins involved in copper trafficking, ranging from ATP-driven transporters to membrane-bound cell surface uptake proteins to small soluble copper chaperones, all appear to be poised to respond to changes in copper status. Although microbial model systems have provided us with vital information on how cellular copper is acquired, distributed, and eliminated, the systems used by these organisms to achieve copper homeostasis in response to changes in copper status are regulated differently from those used by mammals. Whereas regulation of copper-trafficking proteins in lower organisms is mainly at the transcriptional level, mammals seemed to have evolved predominantly post-translational mechanisms to control the activity of these proteins.

Over the last several years we have made significant progress in our understanding of the mechanisms by which copper homeostasis is achieved in higher eukaryotes in response to varying copper availability; however, many important questions still need to be addressed. How do copper uptake transporters at the cell surface sense copper levels? Evidently, Ctr1-independent copper uptake systems exist, yet their identity and regulation in response to changes in copper status is unknown. What role does Ctr2 play in copper trafficking? Are copper chaperones other than CCS regulated by cellular copper concentrations? What is the hierarchy of copper utilization in the cell when copper is abundant or limiting, and what role does copper chaperones play in this process? Answers to these and other questions will be necessary to understand how the diverse systems involved in cellular copper metabolism function together to achieve copper balance under conditions of copper deficiency or overload.

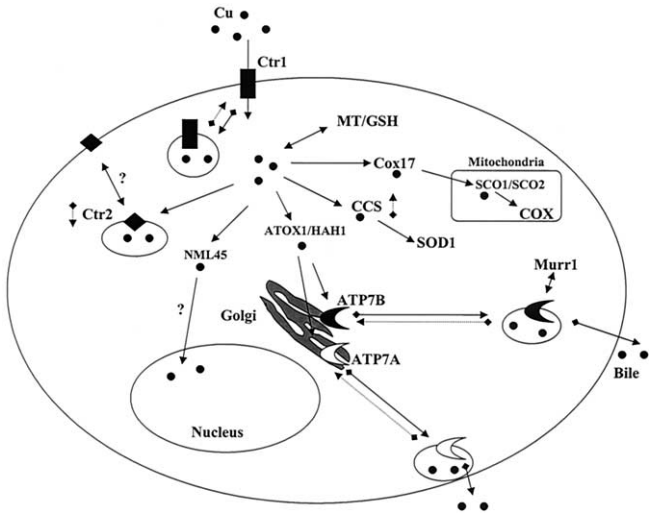

Fig. 1. Copper-trafficking pathways within a mammalian cell. Diagram of a fictitious cell outlining the known copper-trafficking pathways and their regulation in response to copper deficiency (arrows connected by dotted lines) or overload (arrows connected by solid lines) as currently understood. Copper uptake at the cell surface is mediated by Ctr1. When the cell is exposed to high levels of copper, Ctr1 may redistribute to intracellular cytoplasmic vesicles in certain tissues reducing copper influx. Once inside the cell, copper can interact with metallothioneins (MT) and glutathione (GSH). Copper is delivered to specific cellular targets by copper chaperones. CCS inserts copper into Cu/Zn superoxide dismutase (SOD1). In copper-deficient cells, CCS protein level is up-regulated, a response that likely increases the efficiency of copper incorporation into SOD1. Cox17 shuttles copper to the mitochondria for incorporation into cytochrome c oxidase (COX). The mitochondrial proteins SCO1 and SCO2 may function as copper chaperones downstream of Cox17 to insert copper into COX. NML45 may function as a copper chaperone that delivers copper into the nucleus. ATOX1/HAH1 inserts copper into ATP7A and ATP7B. When intracellular copper concentrations are low, ATP7A and ATP7B are predominately localized to the trans-Golgi network. When cellular copper concentrations are elevated, ATP7A redistributes to the cell surface, where it rids the cell of excess copper. In hepatocytes, ATP7B localizes to a cytoplasmic vesicular compartment under conditions of copper overload where it is though to excrete copper into the bile. Murr1 directly interacts with ATP7B and may play a role in the biliary excretion of copper. Ctr2 is localized to cytoplasmic vesicles and may function in intracellular copper storage or mobilization of copper pools. Ctr2 expression is down-regulated with copper deficiency.

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