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Copper entry into human cells: progress and unanswered questions

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Abstract In this brief review we summarize what is known about the role of hCTR1 in mediating the entry of copper into human cells. There is a body of information that clearly identifies this protein as being a major source (though not the only source) of copper entry into human cells, and thus a crucial element of copper homeostasis. However, much remains that is poorly understood and key aspects of the physiological roles of hCTR1 and its regulation are only superficially appreciated. The particular characteristics of a transport process that in vivo involves the binding, transmembrane transport and release of a substrate that is not present in a free form in the intracellular or extracellular compartments poses particular challenges that are not encountered in the transport of more familiar physiologically important metal cations. Thus much of what we have learned about the more commonly encountered transported ions provides an inadequate model for studies of copper homeostasis. In this article we review progress made and identify the major questions that need to be resolved before an adequate description is attained of how copper entry into human cells is mediated and regulated by hCTR1.

Keywords hCTR1 · Copper transport · Copper Homeostasis

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Introduction

Copper is an essential micronutrient in humans, and like many similar metals it is also toxic when allowed to rise above relatively low levels. These simple facts have necessitated the development of sophisticated mechanisms that control and regulate cellular and organismal copper levels. The distribution of copper at the cellular level involves entry and exit mechanisms as well as specific pathways that ensure that this essential enzymatic co-factor is delivered to its required targets. The identification of many of the elements involved in this network has only recently been achieved, but the current picture is doubtlessly incomplete. In this review we will discuss only the copper entry mechanism(s). The major proteins involved in copper uptake in mammalian cells have been identified only in the last 10 years, and we already have some ideas about what they look like, how they function, and what regulatory mechanisms might operate physiologically to ensure the appropriate homeostatic regulation of copper. Although significant progress has been

made we are still in the dark about major aspects of copper regulation. In this brief review we summarize what is known, and we seek to identify the major questions that remain unanswered about the copper entry processes. The article by DeFeo et al in this volume discusses in detail what is known about the structure of hCTR1, the human copper transporter, based on that group's structural studies, which have provided a model of the trimeric organization of this channel-like protein.

The rate of progress in our understanding of the mechanism and role of hCTR1 has been quite rapid. However, there are several intrinsic characteristics of the protein that have slowed progress in this field. These include the low endogenous levels of expression in most human cells and cell lines, the relatively low rates of transport mediated by hCTR1, and the weak antigenicity of most regions of the protein that has hampered numerous efforts to raise specific and high affinity antibodies. Apparent differences in the size and number of proteins in SDS-PAGE gels recognized by anti-hCTR1 antibodies from various groups has clouded the issue of hCTR1 expression patterns and levels in various cells and tissues.

What are the roles of the structural elements of hCTR1?

The first systematic studies on the basis of copper entry into eukaryotic cells came from the laboratory of Klausner in a series of papers in the 1990's that identified the proteins mediating copper uptake by the yeast *Saccharomyces cerevisiae.* (Dancis et al. 1994a, b). These proteins, yCtr1 and yCtr3, were the first members of a widely conserved family subsequently identified as high affinity copper uptake transporters in plants, fungi, mammals and insects. The yeast strains that lack yCtr1 and yCtr3 ($ctr1^{-}ctr3^{-}$) exhibit growth defects characteristic of copper depletion. Functional complementation of these phenotypes was employed to identify hCTR1, and has also been used in structure/function studies of hCTR1 (Zhou and Gitschier 1997; Puig et al. 2002; Aller et al. 2004). The members of this family of proteins contain many potential copper-coordinating residues (histidine, cysteine and methionine), and when examined with hydropathy analyses were predicted to contain three transmembrane segments (Puig et al. 2002; Eisses and Kaplan 2002). The first mammalian transporters identified were from humans (hCTR1) and mice (mCTR1) (Zhou and Gitschier 1997; Lee et al. 2000) and were essentially (92%) identical.

The three transmembrane segment structure implies that the amino and carboxyl termini are on opposite sides of the plasma membrane. Utilizing different approaches, several groups showed that the amino terminus of both yeast and human Ctr1 is on the extracellular side and the carboxyl terminus is in the cytosol (Fig. 1). The extracellular location of yeast CTR1 was shown using protease protection of the C-terminus and inferred from the amino terminal site of glycosylation (Puig et al. 2002). In hCTR1, two consensus sites for N-linked glycosylation in either the amino terminus or the loop were individually mutated to determine which was the site of glycosylation. The site of N-linked glycosylation, and thus the extracellular portion, was in the amino terminus as shown in Fig. 1 at N15 (Eisses and Kaplan 2002; Klomp et al. 2003). In addition, anti-Flag antibody accessibility to amino- and carboxyl-terminal flag tags in permeablized or whole cells was used to confirm that the amino terminus is extracellular and the carboxyl terminus is in the cytoplasm (Eisses and Kaplan 2002).

These studies provide a picture of the topology of the hCTR1 protein that has four major domains (Fig. 1). An extracellular amino-terminal domain of 66 amino acid residues that contains a series of four his-rich and met-rich sequences, an intracellular loop of 46 amino acid residues that is poorly conserved among various species, an intramembrane domain consisting of the three transmembrane segments, and a relatively short carboxyl-terminal tail of 15 amino acids that ends in HisCysHis, a putative metal binding site. Since hCTR1, has only 190 amino acid residues and just three transmembrane segments, it seemed likely that the protein must oligomerize to form the functional transporter. Evidence for oligomeric forms of hCTR1 was

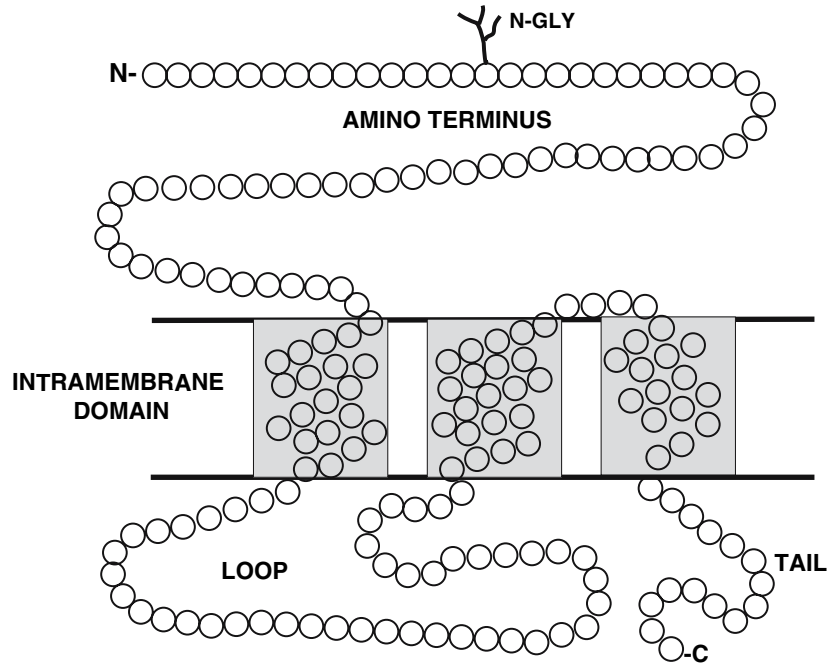

Fig. 1 Topology and domains of hCTR1. N-GLY represents N-linked glycosylation at Asn15. The four domains described in the text are shown

presented in cross-linking experiments and studies showing self-interaction of the amino terminal domain (Lee et al. 2002a; Klomp et al. 2003; Aller et al. 2004). Recently, convincing evidence that clearly shows the homotrimeric organization of hCTR1 in the membrane was provided in the first (low resolution) structure of hCTR1 (Aller and Unger 2006). These results also suggest that a central pore is formed by transmembrane helices in the trimer.

Mutagenic studies of hCTR1 overexpressed in heterologous and human cells have begun to provide a picture of how hCTR1 might provide a permeation pathway for copper across the plasma membrane. Although yeast ctr1^{-}ctr3^{-} complementation assays have proven useful for screening hCTR1 mutants for the complete loss of copper transport activity, the assays cannot provide the necessary quantitation for the evaluation of mutants with more subtle defects in transport. For this purpose, ^{64}Cu uptake assays that allow measurement of the kinetics of copper transport and binding affinity (V_{max} and K_m) have been most informative. In one study, hCTR1 mutants that contained alanine substitutions in four amino-terminal histidine or methionine rich putative copper binding sites (Fig. 2, H1, M1, H2, M2) were tested for complementation of yeast ctr1^{-}ctr3^{-} defects and for ^{64}Cu uptake in HEK293 cells (Puig et al. 2002). The authors showed that only substitutions in methionines M43 and M45 in the MMMMPM sequence at amino acids 40–45 eliminated yeast ctr1^{-}ctr3^{-} complementation and reduced copper uptake in HEK293 cells (see Fig. 2). In agreement with this result, deletion of the first 34 amino acids had little effect on the ^{64}Cu transport activity of hCTR1 in insect cells, but deletion of the first 53 amino acids (which removes MMMMPM) substantially reduced V_{max} and K_m (Eisses and Kaplan 2005b, Fig. 2). In addition, leucine substitution of methionines M150 and M154 shown in Fig. 2 in the second predicted transmembrane sequence abolished yeast complementation and reduced ^{64}Cu uptake in HEK293 and insect cells (Puig et al. 2002).

In the structure/function study of hCTR1 mutants expressed in insect *Sf9* cells, the most striking finding was how few intra-membrane residue substitutions caused any dramatic diminution of transport capacity (Eisses and Kaplan 2005b). V_{max} and K_m values were determined for wild type and twenty-two various truncation and point mutants using ^{64}Cu uptake assays. One of

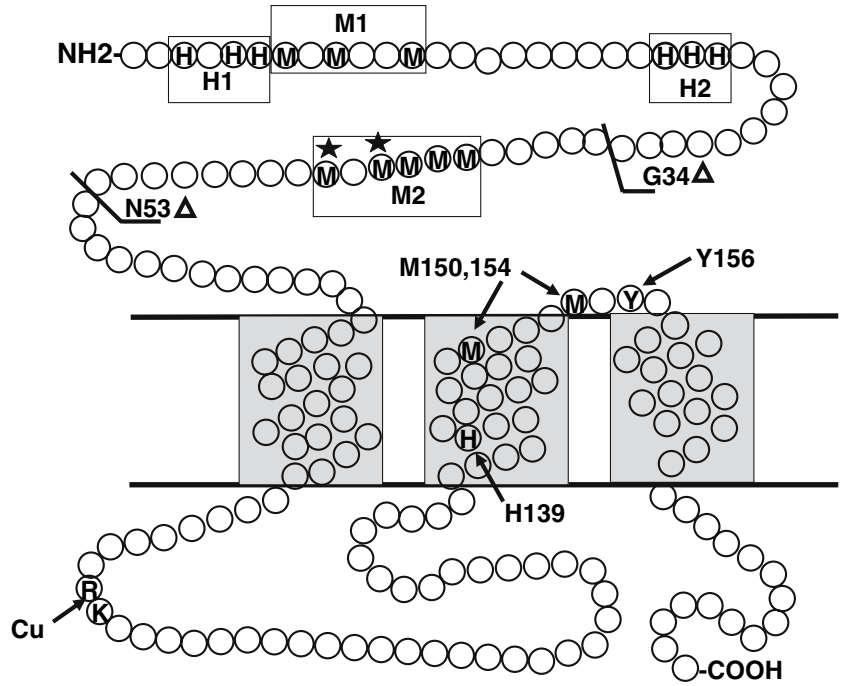

Fig. 2 Features of hCTR1 modified in structure/function experiments. In the amino-terminal domain above 4 boxed Histidine rich (H1 and H2) and Methionine rich (M1 and M2) sequences are shown. Stars indicate M43 and M45. The location of Deletions at G34 and N53 are indicated. In the transmembrane region in the middle, amino acids are shown that affect copper transport, as described in the text. Below, a tryptic-cleave site that is protected by copper binding to hCTR1 (RK) is shown (Eisses and Kaplan 2002)

the most dramatic findings was an increase by about ten-fold in the V_{max} and K_m caused by the substitution of an arginine for a histidine residue (H139R, see Fig. 2) in a position that is lining or protruding into the putative pore region on the cytoplasmic side. Substitution of alanine for tyrosine 156 (Y156A, Fig. 2) at or near the extracellular surface reduced V_{max} substantially, but a number of substitutions of charged or putative copper coordinating residues in transmembrane segments had modest effects. Substitution of M150 and M154 by isoleucine reduced V_{max} three fold but slightly increased K_m.

It thus appears that there are few tight binding sites for copper in the transmembrane domain and that it probably acts as some kind of pore. However, a previous study (Eisses and Kaplan 2002) provided evidence that when copper binds to hCTR1 a conformational change occurs that alters the conformation of the 46 amino acid intracellular loop that is revealed by protection against trypsin proteolysis at the inner surface (see Fig. 2, RK residues in loop). Thus it seems to be an oversimplification to regard hCTR1 as forming a simple pore. Perhaps the protein undergoes conformational changes during its transport cycle. However, it seems likely that while copper is traversing the membrane via hCTR1, there are few, if any sites of tight binding. and that hCTR1 provides a channel-like pathway across the low dielectric region of the membrane. The role of the transmembrane segments and their packing interactions is to provide such a pathway (Aller et al. 2004; Eisses and Kaplan 2005b). A more thorough discussion of many of these points can be found in the article by Defeo et al in this volume.

The essential points that have emerged from studies addressing the movement of copper through the plasma membrane can be summarized as follows. Copper is delivered to the extracellular N-terminal domain of hCTR1 where it is guided into a pore. The N terminus and the pore contains several essential methionine residues involved in this process. Copper binding at the outer surface appears to cause a conformational change in the cytoplasmic loop, but the purpose of this change is not known. The metal ion traverses the pore and exits at the inner surface, perhaps bound at the carboxyl terminus for delivery to its target protein. In contrast to this model, Petris, et al proposed that

hCTR1 was internalized rapidly in the presence of copper concentrations that correlated with transport activation (Petris et al. 2003; Guo et al. 2004). They suggested that hCTR1 may function at least in part as an endocytosing complex with bound copper and hence achieve copper uptake into the cell in a fashion analogous to transferrin-mediated uptake of iron. It now seems that this is unlikely, and that copper uptake is mediated without any necessary internalization of the transporter (Eisses et al. 2005a). The issue of internalization of hCTR1 as a regulatory mechanism is uncertain and is discussed below.

What roles do the amino terminus and intracellular domains play in copper entry? As well as forming a guide system that may serve to focus the copper into the transport pathway, the amino terminus is likely the key element in the delivery of copper to hCTR1 from its partner(s) in the serum. The identification of the serum components that bind copper and deliver it to hCTR1 is the subject of ongoing investigations (Linder 1998; Hassett and Kosman 2005). However, it is clear that whether this agent is a protein, such as ceruloplasmin or serum albumin, or a smaller molecule, it is likely that it's interactions with the exposed extracellular domain of hCTR1 is an important step in the overall uptake process of copper from the serum into the cell. The carboxyl terminus and the intracellular loop, in a similar fashion, must interact with the unidentified copper acceptor is at the cytoplasmic surface following the movement of copper across the membrane. It has been an implicit assumption that when we measure the entry of copper into cells we are measuring a rate-limiting membrane transport step. In fact it has not been established whether there is also kinetic limitation provided by the delivery of copper to hCTR1 from the plasma donor(s), or likewise, from hCTR1 to the intracellular acceptor, or both.

What is the oxidation state of the transported copper?

This is a question that is generally regarded as settled. Most workers in the field believe that the permeating form of copper is Cu(I). It is generally assumed on the basis of the homology between the yeast and mammalian transporters and the evidence in yeast linking the FRE genes to copper uptake (Hassett and Kosman 2005; Martins et al. 1998). In addition, when competitive studies have been performed using other metal ions, there is no inhibition of copper transport by added divalent cations, such as Zn, Mn, Cd, etc, but Ag causes inhibition (Lee et al. 2002a). It has been assumed that Ag would also be transported, although this has not been demonstrated. In addition, the similarity in size of Cu(I) and Ag cations has been taken as good evidence that Cu(I) is the permeant species. Although it is clear from EXAFS studies that copper chaperones such as CCS bind and deliver Cu(I) to their protein targets (Eisses et al. 2000), it is not clear at which precise step in the overall copper homeostasis, from diet to delivery to the target proteins just where the critical redox steps occur, and it is yet unproven that Cu(I) is the only permeant species.

What receives copper from hctr1?

Just as we do not know how hCTR1 receives extracellular copper, we do not know what hCTR1 delivers the transported copper to at its cytosolic surface. Many studies confirm the essential involvement of specific chaperones in delivering copper to its appropriate intracellular sites such as SOD, cytochrome c, ATP7A, ATP7B (Huffman and O'Halloran 2001; Field et al. 2002; Prohaska and Gybina 2004). However, we do not know how these chaperones acquire their copper. One might imagine that each chaperone associates in a protein:protein interaction with an intracellular feature of hCTR1 and that during this interaction copper is transferred in a fashion reminiscent of copper chapoerone:target interactions such as CCS:SOD (Culotta et al. 2006). Alternatively, a common intermediary, such as glutathione or a metallothionine, could accept the copper from hCTR1 and then deliver the metal to various chaperones. There is little if any direct experimental evidence that sheds light on this important question.

What are the functions of hCTR1 post-translational modifications?

Initial studies of hCTR1 using Western blots indicated that post-translational modifications are present in the native protein. Although hCTR1 has a predicted molecular mass of 21 kDa, it migrates in SDS-Page gels as a smear of bands centered at about 35 kDa (Klomp et al. 2002, Maryon et al. in preparation). It appears that both N- and O- linked glycosylation of the extra-cellular amino-terminal domain accounts for most, if not all of the difference in the observed and expected mass of the protein, but the biological role(s) of the glycosylations are unknown at this time. Other potential regulatory modifications, such as phosphorylation, have not been described for hCTR1.

Two laboratories have shown that hCTR1 is modified by N-linked glycosylation on Asn15 (Eisses and Kaplan 2002; Klomp et al. 2003). N-linked glycosylation was inferred from increases in SDS-PAGE mobility of hCTR1 following treatment of cells with the glycosylation inhibitor tunicamycin, or of membranes with PNGase F, which releases N-linked glycans from Asn residues. Mutation of the two Asn residues having consensus sites of N-linked glycosylation (N15 and N112) showed that N15 is the site of polysaccharide addition, consistent with the extracellular location of the amino terminal domain.

The biological function of the N15-linked sugars is uncertain. Expression of N15Q mutant protein in *Sf9* insect cells did not reveal obvious defects in trafficking to the plasma membrane, stability, affinity of copper, or copper transport (Eisses and Kaplan 2002). Similarly, expression of N15D in H441 lung carcinoma cells showed that maturation, stability, and plasma membrane localization of the mutant protein was comparable to that of wild type (Klomp et al. 2003). Although copper transport assays have not been reported for N15Q or N15D mutants expressed in mammalian cells, it seems likely that removal of the N-linked sugars has no striking consequences on the biological function of hCTR1 in tissue culture cells. Whether N-linked glycosylation of hCTR1 plays an important role in intact tissues is an open question.

Recent unpublished work and hints from published experiments shows that native hCTR1 contains O-linked sugars in addition to the N15 linked polysaccharides. Pulse-chase studies of endogenous hCTR1 protein in HeLa cells in the presence of tunicamycin revealed that a 30 kDa protein accumulated that was smaller than mature (N-glycosylated) protein but larger than the unglycosylated precursor (Klomp et al. 2002). In similar pulse chase studies done in H441 cells transfected with epitope-tagged N15D mutant hCTR1, a 30 kDa protein also accumulated (Klomp et al. 2003). We have recently used glycosidase treatment of native wild-type and over expressed mutant proteins to characterize O-linked polysaccharides in the amino terminal domain of hCTR1 (Maryon et al in preparation). Treatment of membranes containing wild-type or N15 mutant hCTR1 proteins with a cocktail of exo-glycosidases that remove O-linked polysaccharides results in a 1–2 kDa decrease in mass of the wild type and mutant proteins on western blots. Digestion with individual glycosidases shows that the terminal residues are sialic acid. Further experiments with protease cleaved hCTR1 and amino-terminal truncation mutants suggest that the O-linked sugars are located within the first 34 amino acids of hCTR1, a region containing 11 serine and threonine residues. The specific serine and/or threonine residues that are sites of O-linked glycosylation are not yet known, nor is it known what biological function, if any, is served by the addition of O-linked polysaccharides.

Aside from the glycosylated amino terminal domain of hCTR1, the cytoplasmic loop and C-terminal tail might be sites of modification by phosphorylation. The cytoplasmic loop and tail contain 10 ser/thr/tyr residues, some of which are reasonable substrates for protein kinases such as PKC. It is conceivable that the smear of bands in SDS gels recognized by hCTR1 antibodies might include polypeptides having different phosphorylation states as well as heterogeneous glycosylation. In any case, our understanding of the nature and role of post-translational modifications of hCTR1 is incomplete.

Is hCTR1 abundance regulated by copper availability?

Copper homeostasis in mammals been shown to be regulated at the level of copper efflux from cells, but it is not clear if copper entry through hCTR1 is also regulated by external copper concentration. Copper efflux occurs via the copper ATPase pumps encoded by the Wilson's disease and Menkes disease genes. Both of these proteins exhibit copper-induced trafficking and redistribution in response to changes in copper abundance (Lutsenko and Petris 2003). It seems reasonable to suppose that the transport activity, or the abundance of hCTR1 in the plasma membrane, or both might also change in response to copper deficit or excess. The absorption of copper by intestinal epithelial cells might also be regulated by hCTR1 abundance or transport activity. Several groups have published work done in cultured cells investigating the regulation of hCTR1 in response to copper depletion or excess.

Studies in cultured cells concerning the regulation of hCTR1 by external copper levels have produced varying results and conclusions. It should be kept in mind that cells encounter little, if any free copper in vivo, since the majority of serum copper is bound to protein (Linder 1998). It is difficult to say precisely what concentration of bound copper is available to hCTR1 in vivo, or whether hCTR1 can accept Cu(I) and Cu(II), both of which are bound to serum proteins. Furthermore, immortalized cell lines used in these studies may respond differently than analogous cells in living tissues.

Published reports describing the effects of elevated copper on the localization of hCTR1 differ in their conclusions. Klomp et al. examined the localization of hCTR1 using an antibody raised against the N-terminal domain (Klomp et al. 2002). The distribution of hCTR1 staining with this antibody varied in different cell lines, but membrane localization did not change in HeLa or Caco-2 cells when the cells were pre-treated with either the a copper chelator BCS, or 400 μM copper. In these same cells the Menkes disease protein ATP7A moved in response to 400 μM copper from the trans-golgi network to a diffuse staining pattern near the plasma membrane, as described (Lutsenko and Petris 2003). Another report concluded from western blot and confocal analysis that hCTR-1 protein levels were unchanged in Caco-2 cells grown on transwells when grown in low to high (94 μM) copper, but that some of the hCTR1 signal appeared to move inside the cells (Bauerly et al. 2004). It should be noted that the antibody used in this study recognized peculiar sized bands of 40–48 kDa in addition to the smear of bands at 35 kDa observed with anti-hCTR1 antibodies in other studies.

Petris et al. reported a striking effect on hCTR1 localization following incubation in 100 μM copper (Petris et al. 2003). They observed a rapid redistribution of over-expressed epitope-tagged hCTR1 from the plasma membrane to interior vesicular compartments in HEK293 and CHO cells. Surprisingly, hCTR1 appeared to be internalized within 10 min, and the effect saturated between 5 and 20 μM copper. hCTR1 internalization was inferred by detecting the internalized anti-myc antibodies rather than following the hCTR1 protein. Internalization appeared to proceed through a clathrin-dependent process into the same compartment as endocytosed transferrin. The myc-tagged hCTR1 protein rapidly disappeared on western blots following a two-hour treatment of 20 or 100 μM copper in cyclohexamide-containing media, which was interpreted as copper-elicited degradation of hCTR1. The authors speculated that hCTR1 may admit copper into the cell by an endocytotic pathway in which the hCTR1 protein is destroyed and copper bound to the protein is released and subsequently delivered to it's target.

A forth published report by Eisses and Kaplan describes studies of over expressed hCTR1 in *Sf9* insect cells and endogenous hCTR1 in HEK293 cells. In insect cells, 100 μM copper pre-treatment had no effect on ^{64}Cu uptake (over 70 min), or on plasma membrane levels of hCTR1 as judged by western blot. In HEK293 cells, 100 μM copper pretreatment had no effect on the kinetics of ^{64}Cu uptake over 80 min, and little or no loss of the hCTR1 protein from plasma membrane fractions was observed. The copper uptake was sensitive to knockdown of hCTR1 using RNAi. Confocal imaging with anti hCTR1 antibodies revealed no obvious change in the plasma membrane locali-

zation of endogenous hCTR1 following treatment of HEK cells with 100 μM copper.

Finally, one recent published study used anti-CTR1 antibodies to evaluate tissue-specific expression of CTR1 in mouse tissues from animals having normal or copper deficient diets (Kuo et al. 2006). Mice suckled by copper deficient dams showed elevated mCTR1 staining in kidney, intestine (duodenum), and in brain tissue. Quantitation of these effects was difficult with the methods used. In addition, the antibody in this study recognized multiple bands not clearly related to hCTR1.

It is unclear why different groups have observed such divergent effects of copper on hCTR1behavior in the plasma membrane; it is possible that over-expressed, myc-tagged hCTR1 behaves differently than endogenous proteins. It is also conceivable that the epitope tag might be lost by some kind of copper stimulated proteolytic cleavage without the loss of the hCTR1 transporter. Such an alternative might explain the discrepancies between experiments using anti-myc-antibodies and experiments using anti-hCTR1 antibodies or ^{64}Cu uptake. It seems difficult to reconcile the 10 min time scale reported for copper stimulated internalization-degradation of myc-hCTR1 (Petris et al. 2003) with the linear ^{64}Cu uptake kinetics over 60 min. observed in both insect and human cells (Lee et al. 2002a; Eisses and Kaplan 2002; Eisses et al. 2005a). Furthermore, internalization of anti-myc antibodies and loss of myc-hCTR1 was maximal between 5 and 20 μM added copper (Petris et al. 2003). In human and insect cells expressing endogenous or over-expressed hCTR1, ^{64}Cu uptake reaches a maximum between 5 and 20 μM copper and then stays constant at higher copper concentrations (Lee et al. 2002a; Eisses and Kaplan 2002; Eisses et al. 2005a). One would predict from the internalization-degradation model that prolonged incubation in high copper or a steady increase in copper concentration would result in decreased ^{64}Cu uptake as the transporters are rapidly removed from the plasma membrane and degraded.

It is not yet clear how hCTR1 abundance, localization, or transport activity changes in response to the extracellular copper concentration. Also unknown is whether copper levels inside the cell might modulate the activity, trafficking, or localization of hCTR1.

What mediates hCTR1-independent Copper entry?

Unequivocal evidence that CTR1 is not the only protein capable of mediating copper entry into mammalian cells was provided by Lee et al., in which embryonic cell lines from mCTR1 knock-out mice were isolated and characterized (Lee et al. 2002b). In spite of the fact that mCTR1 –/– animals die in mid gestation at E10-E12 (Kuo et al. 2001; Lee et al. 2001), cell lines derived from the embryos grew well without added copper. The mCTR1 –/– cells exhibited about 20–30% of the copper uptake activity observed in wild-type cells derived from littermate embryos. This CTR1-independent copper transport activity saturated with a K_m for copper of 10 μM, which was 10 fold higher than the K_m previously measured for hCTR1 (1 μM). The mCTR1 –/– cells also had a different profile of copper uptake inhibition by metals other than Cu, showing modest inhibition by Fe, Mn, Cd, and most strikingly by Zn. In an earlier study, copper transport in cells expressing endogenous hCTR1, or cells over-expressing mCTR1 or hCTR1 showed transport inhibition by Cu and Ag, but not Zn or other divalent metals (Lee et al. 2002a).

The identity of the CTR1- independent copper transport activity is unknown. It is quite possible that Cu(II), rather than Cu(I) is transported by this system, based on divalent metal inhibition. The transporter DMTI, well known for its role in iron uptake, has a broad specificity of metal substrates and is widely expressed (Garrick et al. 2003), although iron was only a weak competitor of mCTR1-independent copper uptake. Based on the inhibition of transport by zinc, zinc transporters such as Zip1 are candidate proteins for CTR1-independent copper transport. As present there is no data to support a role for Zip1 in copper uptake. The embryonic lethal phenotype of mCTR1 –/– animals shows that in mammals, CTR1 is an essential entry portal for the delivery of copper to it's targets, at

least in some cell types. It is less certain that CTR1 is the only important route of copper acquisition in other cells or from the diet. mCTR1 –/– mice die before these issues can be investigated. DMT1 and/or unidentified proteins must also function to acquire copper in mCTR1 –/– cells, but the biological significance of CTR1-independent copper entry in mammals is unknown at his time.

Conclusion

In summary, almost ten years have passed since the cloning of hCTR1. Despite significant progress in understanding the structure, the mechanism of transport, and the essential biological role of hCTR1 in copper homeostasis, we do not understand many aspects of this unusual trimeric transporter. For instance, how does CTR1 accept and deliver copper from donor and accepter proteins, and what are these proteins? How is hCTR1 is regulated by the concentration of copper outside (or inside) the cell? How does hCTR1 interact with other components of the network to maintain copper homeostasis? In the next decade, answers to these and other questions will provide a better understanding of hCTR1 and of copper homeostasis in humans.

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