Molecular Characterization of Copper Uptake into Eukaryotic Cells Mediated by Human Copper Transporter 1

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ABSTRACT

Copper is an essential element that acts as a cofactor in many enzymes. Cellular regulation of uptake, redistribution and export of copper is vital for the proper function of many physiological processes and reactions. Several disease pathologies have a link to non physiological concentrations of copper in the body. Proteins have been identified that mediate the proper uptake, distribution and excretion of copper from cells.

In this dissertation, the process of cellular copper uptake was explored through the study of the human Copper transporter 1 (hCtr1). hCtr1 is a high affinity copper uptake protein that is essential for embryonic development and proper incorporation of copper into cells. hCtr1 is believed to mediate the transport of Cu (I) across the plasma membrane in many cells. Identified in 1997, hCtr1 was initially shown to function as a copper uptake protein by the functional complementation in copper deficient yeast. The ability of hCtr1 to complement yeast copper deficiency underscores the similarity of this family of proteins. hCtr1 is a relatively small protein containing 190 amino acid residues. It has three putative membrane spanning regions as shown by hydropathy analysis. The amino terminus is glycosylated and is located in the extracellular space while the carboxyl terminus is in the cytoplasm of the cell. It has been shown to form a stable complex consisting of multiple hCtr1 monomers.

The protein has relatively few identical residues when compared to Ctr1 proteins from other species, however, results from this dissertation as well as reports from others provide insights into a conserved mechanism for uptake of copper. We show that hCtr1 mediates saturable copper uptake with a Km of ~6-8 μM in heterologous expression systems. The protein contains few potential copper binding residues. Two methionine

repeats exist in the protein and have been shown to be vital for efficient copper uptake. One is found in the amino terminus (residues 40-45) and the second is found in the second transmembrane domain (residues 150-154). While the two cysteines present in the protein do not appear to bind copper, they may play a structural role. We have identified other residues that affect the ability of this protein to transport copper in an efficient manner. Residues in the third transmembrane segment as well as residues in the carboxyl terminus may play an important role in the assembly of hCtr1 molecules to form a functional complex. We have also shown that copper induces a conformational change in the cytoplasmic loop between transmembranes 1 and 2.

Many of the proteins that are involved in the efficient delivery of copper to target enzymes are tightly regulated to ensure copper availability and to reduce copper toxicity. An example of this regulation in humans is the relocalization of copper export proteins (i.e. Menkes disease protein) to the plasma membrane where they remove excess copper from the cell. Our results show that hCtr1, unlike yCtr1 but similar to yCtr3, is a stable protein at the plasma membrane and it does not seem to be regulated post-translationally in response to high extracellular copper concentrations.

Chapter 1

Introduction

1.1 Importance of Copper Homeostasis

Copper is an essential cofactor for many cellular enzymes. The importance of copper ions in humans is demonstrated by its requirement for cellular respiration, iron homeostasis, pigment formation, neurotransmitter biosynthesis, peptide biogenesis, connective tissue production and antioxidant defense. In several neurological disorders and neurodegenerative conditions such as Alzheimers disease, amyotrophic lateral sclerosis, Wilson disease and prion diseases copper either has a definitive role or has been suggested to be involved. The link between copper and these human diseases has raised the awareness of the importance of metal homeostasis. How the delivery of copper contributes to the proper or improper function of copper containing enzymes has become a significant field of research.

Studies of copper metabolism in humans and the regulation of the molecules involved in this process have seen remarkable growth in the last ten years. These studies were initiated by the discovery of human copper transporting ATPases, ATP7A and ATP7B. The protein products of these genes are mutated in two hereditary disorders of copper homeostasis, Menkes disease and Wilson disease respectively. These two proteins are involved in balancing copper concentrations within cells and mutation of these transporters causes copper related toxicosis.

The importance of understanding copper homeostasis is underscored by the number of proteins that utilize copper in various metabolic processes. Copper preferentially binds to atoms such as nitrogen, oxygen and sulphur, which are available for metal binding by the side chains of amino acid residues methionine, cysteine, histidine, aspartate and tyrosine as well as other less common copper coordinating

residues, glutamate, threonine, serine, glutamine and asparagine. These amino acid residues play important roles as ligands in copper binding and therefore are found in active sites of enzymes that use copper as cofactors for catalytic reactions. A short list of these proteins and processes are found in Table 1.

The ability to maintain the proper levels of copper in the cell must be tightly regulated as too much copper can be very toxic for cells but also too little copper inhibits many essential processes. The cytotoxicity of copper has made it necessary for cells to evolve specialized pathways for the handling of copper ions. It has been estimated that less than 1 free copper ion exists per cell (Rae, Schmidt et al. 1999), which suggests that intricate regulatory mechanisms exist to ensure that copper entering the cell is bound to proteins or other cellular components in order to limit the potential toxic effects of copper.

Wilson disease patients suffer from copper accumulation in the liver and the brain, resulting in liver toxicity and neurological disease underscoring the toxic nature of excess copper. Copper can exist in either the reduced Cu (I) or oxidized Cu (II) state and can therefore act as an important co-factor in a number of redox reactions. However, because of this potent redox capability, copper can also generate highly reactive oxygen species (ROS), including hydroxyl radicals known to cause damage to proteins, lipids and DNA.

Toxic effects can occur, in addition to ROS generation, when copper displaces other metal cofactors from their natural ligands in key cellular signaling proteins. Therefore copper has been suggested to play a role in many disease processes. In some of these diseases, the role of copper is evident and the gene defect has been identified,

however others have not been characterized and the role of copper ions in the disease process is unclear. A brief description of a few of these diseases in which copper plays a putative role, will help to demonstrate the range of effects and the potential toxic effects copper can have in the organism.

1.2 Copper Associated Diseases

1.2.1 *Menkes Disease*

Menkes disease is an X-linked inherited disorder that is caused by mutations in the ATP7A gene (Mercer, Livingston et al. 1993; Vulpe, Levinson et al. 1993). Affected patients suffer from systemic copper deficiency, leading to neurodevelopmental delay, kinky hair, fragile bones and aortic aneurisms (Martins, Goncalves et al. 1997). Loss of function of this protein in affected individuals results in failure of copper transfer across the placenta, gastrointestinal tract, and blood-brain barrier, with resultant copper deficiency in the developing fetus. Classical Menkes disease is usually lethal by age three, but there are two milder allelic variants of the disorder, called occipital horn syndrome and mild Menkes disease (Tsukahara, Imaizumi et al. 1994). A spontaneous mutation has occurred in mice, the mottled/brindled mouse mutant, which is considered the closest animal model of Menkes disease. These mice display a mutation in the orthologous ATP7A gene (Grimes, Hearn et al. 1997).

1.2.2 *Wilson's Disease*

Wilson's disease is an autosomal recessive genetic disorder, caused by mutations in the ATP7B gene (Bull, Thomas et al. 1993; Tanzi, Petrukhin et al. 1993; Yamaguchi, Heiny

et al. 1993). The Wilson's disease gene has been identified by positional cloning strategies and has sequence homology to the Menkes disease gene (Bull, Thomas et al. 1993; Petrukhin, Fischer et al. 1993; Tanzi, Petrukhin et al. 1993). Patients with Wilson's disease show a range of clinical symptoms including chronic hepatitis and cirrhosis resulting from toxic accumulation of copper in the liver. Concomitant with a defect in biliary copper excretion, patients can also present with neurological damage particularly affecting speech and movement, and leading to psychiatric disturbance in a number of cases, because of toxic copper accumulation in the brain. Two animal models for Wilson's disease, containing either a deletion or a mutation in the gene homologous to human ATP7B, have been identified. The Long-Evans rat with a cinnamon-like coat color is an inbred mutant strain with spontaneous hepatitis (Takeichi, Kobayashi et al. 1988; Wu, Forbes et al. 1994). A second model was identified as well; lactating homozygous dams of the toxic milk mouse mutant strain produce copper-deficient milk which is lethal to their pups(Theophilos, Cox et al. 1996). Since this time knockout mice have been generated by several groups.

Other diseases with a copper link exist that are not caused by mutations in proteins that are responsible for copper transport. These include COX deficiency, Amyotrophic lateral Sclerosis, Alzheimer's disease and Prion diseases. Each of these diseases are associated with changes in proteins that contain copper as a cofactor and the proposed disease mechanism has been suggested to have a link to improper copper binding or altered copper catalysis.

1.2.3 *COX deficiency*

Mammalian cytochrome c oxidase (COX) is a multimeric enzyme of the inner mitochondrial membrane whose 13 subunits are encoded by both the mitochondrial and nuclear genomes. The enzyme contains two iron-clusters and three copper atoms, and catalyzes the reduction of molecular oxygen by reduced cytochrome c, the terminal step in the respiratory chain. COX deficiency can be found in neonatal, infantile and late onset diseases that can present with a wide spectrum of multisystemic, neurological and muscular symptoms (Shoubridge 2001). Autosomal recessive mutations in several of the nuclear genes encoding accessory factors involved in the assembly of the holoenzyme COX have been identified and copper has a role in some of these. Copper is involved in diseases caused by mutations in SCO1 (tubulopathy with encephalomyopathy) and SCO2 (fatal hypertrophic cardiomyopathy with encephalopathy) (Papadopoulou, Sue et al. 1999; Jaksch, Ogilvie et al. 2000; Valnot, Osmond et al. 2000). SCO1 and SCO2 are homologous proteins, found in the mitochondria that are presumably active in copper incorporation into COX, since COX activity in myoblasts from SCO2-patients is completely rescued by addition of 300μM copper-histidine to the culture medium (Jaksch, Paret et al. 2001).

1.2.4 *Amyotrophic Lateral Sclerosis*

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease with a sporadic and an inherited form, and is characterized by selective degeneration of motor neurons, leading to progressive muscle weakness, atrophy, and death often within 3 years of the onset of symptoms in the spinal cord, brainstem and motor cortex. A number of familial cases of ALS have been associated with defects in the Cu/Zn superoxide dismutase

protein. Cu/Zn superoxide dismutase plays a major role as a cellular antioxidant by converting superoxide radicals into hydrogen peroxide and molecular oxygen, and it requires copper in its active site for this enzymatic activity. Heterozygous mutations in SOD have been linked to this disease (Deng, Hentati et al. 1993; Rosen, Siddique et al. 1993). The neurotoxicity associated with mutant Cu/Zn superoxide dismutase is not well understood, but is thought to occur by a gain-of-function mutation, consistent with the autosomal dominant inheritance (Gurney, Pu et al. 1994; Ripps, Huntley et al. 1995; Wong, Pardo et al. 1995; Reaume, Elliott et al. 1996; Ho, Gargano et al. 1998). Many theories exist as to the mechanism of this toxicity, a recent suggested mechanism centered on mutations that destabilized protein folding causing subsequent formation of cytotoxic protein aggregates.

1.2.5 *Other copper related diseases*

In several other complex disorders, a putative role for copper has been postulated. An example of such a disease is Alzheimer's disease (AD), which occurs in a sporadic and an inherited form. AD is characterized by progressive neuronal dysfunction, reactive gliosis, and the formation of copper and iron-containing amyloid plaques in the brain (Smith, Richey Harris et al. 1997; Sayre, Perry et al. 2000). Two peptides which play a central role in AD, the amyloid precursor protein and a proteolytic breakdown product of APP called Aβ, can bind Cu(II) and reduce it to Cu(I) *in vitro* (Hesse, Beher et al. 1994; Multhaup, Schlicksupp et al. 1996; Atwood, Moir et al. 1998; Cherny, Legg et al. 1999; Huang, Atwood et al. 1999; Huang, Cuajungco et al. 1999). The cause of the neuronal cell loss in AD is unclear but may be related to increased oxidative stress from excessive

free radical generation, possibly as a result of defects in copper homeostasis (Smith, Richey Harris et al. 1997; Martins, Jensen et al. 1998; Bush 2000; Sayre, Perry et al. 2000).

Prion encephalopathies (Mad cow disease and Creutzfeld-Jakob disease) are also linked to aberrant copper mechanisms. Prion diseases are characterized by a rapid progressive dementia and cerebellar ataxia resulting from neuronal spongiform degeneration and astrocytic gliosis, and are caused by the accumulation of a post translationally modified form (PrP^{Sc}) of the normal cellular prion protein (PrP^{C}) (Prusiner 1997). PrP^{C} is able to bind copper and is mostly expressed by neurons and by glia (Kretzschmar, Prusiner et al. 1986; Hornshaw, McDermott et al. 1995; Moser, Colello et al. 1995; Miura, Hori-i et al. 1996). Cerebellar cells from mice deficient in PrP^{C} reveal increased sensitivity to the toxicity of copper containing salts (Bueler, Fischer et al. 1992), marked decrease in membrane copper content and decreased activity of Cu/Zn superoxide dismutase (Brown, Qin et al. 1997; Brown 1998), suggesting a role of PrP^{C} in copper homeostasis.

Taken together, it is clear that some disorders are caused by an imbalance of cellular copper concentration. The role of copper in the etiology of many other disorders is not yet well defined. Further, several probably sporadic disorders of copper homeostasis have been described, for which the specific causes is unknown. The identification of mutated genes has shed some initial light on the regulation of copper homeostasis, but it is obvious that many components of the copper homeostasis pathways are still unidentified. Therefore, fundamental research of copper transport mechanisms in

healthy mammals is essential for better comprehension of copper –related disorders and for the development of treatment strategies.

1.3 Cellular Copper Transport Mechanisms

Insights into the identification of cellular components that play a key role in copper homeostasis were derived from studies in model organisms such as bacteria and yeast. Many of the identified components are structurally and functionally conserved between species, emphasizing the importance of copper homeostatic mechanisms (figure 1.1). To maintain a critical balance between copper necessity and toxicity, cells have developed sophisticated mechanisms that rapidly respond to variations in environmental copper concentrations. Construction of knockout mouse strains has further demonstrated the essential nature of copper systems. The uptake and intracellular compartmentalization or buffering of copper is strictly regulated in single cell organisms such as yeast. In complex organisms such as mammals, this balance is achieved not only by regulating copper at a cellular level, but also seems to be processed and utilized by specific target organs and tissues (Frieden 1986; Vulpe and Packman 1995; Linder and Hazegh-Azam 1996).

Copper is an essential dietary nutrient. The adult human contains about 80-100mg copper, much of this in the liver (10mg), brain (8.8mg), blood (6 mg), skeleton (including blood marrow; 46 mg), and skeletal muscle (26mg) (Halliwell and Gutteridge 1985; Linder and Hazegh-Azam 1996; Harris 2003). This information is represented schematically in figure 2, showing the major players for both copper absorption and distribution in humans at an organ level. In developed countries, the average daily adult

diet contains about 5mg of copper, and somewhere between 0.6-1.6 mg per day is absorbed via the stomach and duodenum, where extra-cellular pH is relatively low compared to other parts of the intestine. Mechanisms that transport copper into and out of these duodenal cells have been identified (Crampton, Matthews et al. 1965; Van Campen and Gross 1968; Linder and Hazegh-Azam 1996; Pena, Lee et al. 1999). Two candidate proteins responsible for the copper uptake are expressed at the brush border of the intestine. The first is the divalent metal transporter 1 (DMT1), also known as natural resistance associated macrophage protein 2 (Nramp2), or divalent cation transporter 1 (DCT1) (Fleming, Trenor et al. 1997; Gunshin, Mackenzie et al. 1997; Fleming, Romano et al. 1998; Tennant, Stansfield et al. 2002). DMT1 is an iron transporter that can also act as a copper transporter, but reports about the extent of the involvement in intestinal copper uptake are inconclusive (Gunshin, Mackenzie et al. 1997; Tandy, Williams et al. 2000; Arredondo, Munoz et al. 2003). A second candidate is human copper transporter 1 (hCtr1), which has been shown to have copper transport activity, selective for Cu (I) (Zhou and Gitschier 1997; Kuo, Zhou et al. 2001; Lee, Prohaska et al. 2001; Lee, Pena et al. 2002). Since Cu is largely found as Cu (II) in the diet, copper would first have to be reduced to Cu (I) to be transported via hCtr1. Numerous proteins are potentially capable of reducing copper including endogenous plasma membrane reductases or dietary components such as ascorbate (McKie, Barrow et al. 2001; Knopfel and Solioz 2002). Most copper transfer across the basolateral membrane to blood and interstitial fluid is likely to involve ATP7A, since it is highly expressed in enterocytes, and a defect of this protein results in severe copper deficiency in humans (Chelly, Tumer et al. 1993; Mercer, Livingston et al. 1993; Vulpe, Levinson et al. 1993).

The newly absorbed copper is transported via the portal circulation to the liver (Sternlieb and Scheinberg 1972). Only a few hours following a dose of radioactive copper is required for 95% of the isotope to be removed from the circulation by the liver, and within the next 24 hours, isotopic copper can be measured in ceruloplasmin in the plasma (Hellman and Gitlin 2002). Ceruloplasmin is a ferroxidase, which is synthesized in hepatocytes and is secreted as a single polypeptide chain of 1046 amino acids with six atoms of copper bound to it (Osaki and Johnson 1969; Roeser, Lee et al. 1970). A major role of ceruloplasmin in intestinal copper absorption, uptake in the liver or biliary excretion has been difficult to confirm since aceruloplasminemic patients exhibit no copper imbalance (Yoshida, Furihata et al. 1995; Harris, Klomp et al. 1998). Estimates suggest that the majority of plasma copper is bound to ceruloplasmin and less to serum albumin and histidine (Linder and Hazegh-Azam 1996). Plasma copper is filtered and reabsorbed in the kidney with some excess copper being excreted in the urine; higher than normal urine copper content is a diagnostic test for Wilson's disease. One of the major pathways for the removal of copper from the body relies on the Wilson's disease protein in the liver. Excess copper is excreted to bile by the Wilson's disease protein via the canalicular plasma membrane. Copper appears in the bile and as a result, equivalent amounts of copper are absorbed each day by the gastrointestinal tract and excreted into the bile (Tao and Gitlin 2003). Since the liver provides the most physiologically relevant mechanism for copper excretion, it is thought to be the central organ for regulating copper levels in the body. At a cellular level, regulation of copper homeostasis is carried out via a complex set of processes.

1.3.1 *Cellular copper transport: export*

In 1993 the homologous copper-export proteins ATP7A and ATP7B, affected in patients with Menkes disease and Wilson's disease, respectively, were identified. These proteins were shown to be homologous to proteins in a number of other species from bacteria to mammals. Similar defects in these two proteins' ability to efflux copper gives rise to two distinctly different diseases is spite of the proteins being similar structurally and functionally (Payne and Gitlin 1998). This difference in disease manifestation is likely due to differences in tissue expression of these two proteins. ATP7A mRNA is expressed in enterocytes and the endothelium of the blood brain barrier and many other tissues (Vulpe, Levinson et al. 1993; Murata, Kodama et al. 1997) while ATP7B is expressed predominantly in the liver and at lower levels in the brain, kidneys, placenta, and the heart (Tanzi, Petrukhin et al. 1993; Vulpe, Levinson et al. 1993; Kuo, Gitschier et al. 1997; Murata, Kodama et al. 1997; Saito, Okabe et al. 1999). Within the brain, cell specific expression of these two proteins has been reported and the expression of these two proteins changes during development (Barnes, Tsivkovskii et al. 2005).

ATP7A and ATP7B belong to a large family of cation-transporting P-type ATPases, a group of integral membrane proteins that translocate ions across cell membranes, using the energy of ATP hydrolysis (Lutsenko and Kaplan 1995; Scarborough 1999). These two proteins have characteristic CPC (cysteine-proline-cysteine) motif in one of the eight transmembrane regions, which is thought to be involved in translocation of copper across membranes in the cell (Lutsenko and Kaplan 1995; Solioz and Vulpe 1996). In their N-termini, these P-type ATPases uniquely have six repetitive sequences, each carrying the metal binding motif MTCXXC, which can

bind one copper atom in the reduced Cu (I) form per domain (Lutsenko, Petrukhin et al. 1997; DiDonato, Narindrasorasak et al. 1999; Jensen, Bonander et al. 1999). The copper metal-binding domains are arranged in a ferrodoxin-like $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ fold, and the two cysteine residues are the major ligands coordinating the copper atom (Huffman and O'Halloran 2001; Ralle, Lutsenko et al. 2003). Currently it is not known how many copper atoms are transported per molecule of hydrolyzed ATP. It is also unclear whether copper transport by ATP7A or ATP7B is electrogenic and whether or not a counter ion is involved. Similarly, it remains to be determined how copper is released from the transporters after the ion crosses the lipid bilayer.

To protect cells against toxic copper concentrations, ATP7A and ATP7B proteins undergo copper-responsive changes in intracellular trafficking. Under low copper conditions, both proteins are localized in the trans-Golgi network where they deliver copper to secreted cupro-proteins (like ceruloplasmin) (Petris, Mercer et al. 1996; Yamaguchi, Heiny et al. 1996; Dierick, Adam et al. 1997; Hung, Suzuki et al. 1997). In the presence of elevated copper (20-50 μM copper), they are redistributed to either intracellular vesicles located near the plasma membrane or the plasma membrane, where they are thought to excrete copper to the extracellular milieu and are phosphorylated by a kinase (Petris, Mercer et al. 1996; Yamaguchi, Heiny et al. 1996{Vanderwerf, 2001 #250; Payne and Gitlin 1998; Roelofsen, Wolters et al. 2000). The role of this phosporylation is not known at this time. A conserved di-leucine motif near the C-terminus of ATP7A mediates its endocytosis back to the Golgi apparatus in a reversible process, independent of protein synthesis (Petris, Camakaris et al. 1998; Francis, Jones et al. 1999; Petris and Mercer 1999). Although the exact molecular mechanisms of

ATP7A/B trafficking are unknown, copper –dependent phosphorylation on a non-catalytic serine residue of the ATPases and a member of the Rho GTPase family named Cdc42, are involved (Vanderwerf, Cooper et al. 2001; Cobbold, Ponnambalam et al. 2002). The regulated localization of the Wilsons and Menkes disease proteins provides a way of removing excess copper from the cell in response to cellular copper content.

1.3.2 *Cellular copper transport: intracellular distribution*

Once copper is inside the cell, free copper ions are virtually undetectable (Rae, Schmidt et al. 1999), but are transiently associated with small copper-binding proteins, denoted copper chaperones, which distribute the copper to specific intracellular destinations (Figure 1.1). Thus far, three copper chaperones have been identified through genetic studies in yeast. One of the copper chaperones, ATOX1, delivers copper to the copper transporting ATPases ATP7A/B in the trans-Golgi network (Klomp, Lin et al. 1997; Pufahl, Singer et al. 1997). A second chaperone, COX17, is responsible for delivering copper to the mitochondria for incorporation into cytochrome c oxidase (COX) (Amaravadi, Glerum et al. 1997). The third copper chaperone, copper chaperone for superoxide dismutase (CCS), is responsible for copper incorporation into Cu/Zn superoxide dismutase in the cytosol and in the mitochondrial intermembrane space (Culotta, Klomp et al. 1997; Valentine and Gralla 1997).

ATOX1 was originally identified as a copper dependent suppressor of oxidative damage in yeast lacking superoxide dismutase (Lin and Culotta 1995). Structural predictions based on the presence of ferredoxin-like folds in both ATOX1 and ATP7A/B, suggest copper is transferred via a ligand-exchange reaction involving two- and three-

coordinate intermediates between cysteine ligands in the CXXC motifs on ATOX1 and the recipient ATP7A/B proteins (Pufahl, Singer et al. 1997; Hamza, Schaefer et al. 1999{Walker, 2004 #77; Larin, Mekios et al. 1999; Rosenzweig, Huffman et al. 1999; Wernimont, Huffman et al. 2000; Walker, Tsivkovskii et al. 2002; Walker, Huster et al. 2004). In cells isolated from Atox1$^{-/-}$ mice, copper dependent sub-cellular trafficking of ATP7A is markedly impaired (Hamza, Prohaska et al. 2003). Further, it was shown that ATOX1 stimulates the catalytic activity of ATP7B (Lutsenko, Tsivkovskii et al. 2003). Taken together, these data indicate that ATOX1 function is not restricted to only copper-delivery at the Golgi apparatus, but may also be involved in regulation of the copper excretion process.

Cu/Zn superoxide dismutase is an enzyme that contains both copper and zinc ions. This enzyme is active as a dimer and acquires its copper via CCS. It is located in the cytosol and the inter-membrane space of mitochondria (Casareno, Waggoner et al. 1998; Corson, Strain et al. 1998; Wong, Waggoner et al. 2000; Okado-Matsumoto and Fridovich 2001; Sturtz, Diekert et al. 2001). The CCS protein comprises three distinct functional regions. The N-terminal region (domain I) is similar in structure to that of ATOX1, containing a MTCXXC copper binding motif structured in a ferredoxin-like fold (Lamb, Wernimont et al. 1999; Lamb, Wernimont et al. 2000). Domain II has an overall structure similar to Cu/Zn superoxide dismutase and residues involved in Cu/Zn superoxide dismutase homodimerization are conserved in CCS, allowing heterodimer formation between CCS and SOD (Casareno, Waggoner et al. 1998; Lamb, Wernimont et al. 1999; Schmidt, Rae et al. 1999; Schmidt, Kunst et al. 2000; Rae, Torres et al. 2001).

Domain III contains a conserved CXC motif that is required for copper transfer into Cu/Zn superoxide dismutase.

COX is a key integral membrane mitochondrial enzyme in the respiratory chain, which requires a total of three copper ions to be inserted into two of its 13 subunits (Tsukihara, Aoyama et al. 1995). The exact mechanism of copper insertion is unknown, but a substantial role has been assigned to COX17; a gene product identified by complementation analysis of the respiratory defect in yeast (Amaravadi, Glerum et al. 1997). In yeast, Cox17p is located in the cytosol and the mitochondrial intermembrane space, prompting a model in which copper-mediated cytosolic yCox17p would translocate into the intermembrane space to deliver copper to COX (Beers, Glerum et al. 1997). In the same organism, copper incorporation into COX also requires the presence of Sco1p, a mitochondrial inner membrane protein, and also the homologous protein Sco2p may play a role in COX activation (Schulze and Rodel 1989; Glerum, Shtanko et al. 1996).

In addition to these copper chaperones, metallothioneins (MT) are present in the cytosol. MTs are low molecular weight (~7kDa) proteins with a high metal (7-20 atoms/molecule) and sulphur (~30% cysteine residue) content. The major function of MT is thought to be detoxification of metals and storage of essential trace metals. MT genes are universally induced at the transcriptional level by metals and a variety of stress conditions such as reactive oxygen species, hypoxia and irradiation (Yagle and Palmiter 1985). Heavy metal-associated induction occurs at copper concentrations of 5μM (Hidalgo, Garcia et al. 1994), and is mediated by metal-responsive promoter elements

(MREs) and the MRE-binding transcription factor MTF-1 (Brugnera, Georgiev et al. 1994; Heuchel, Radtke et al. 1994).

1.3.3 *Cellular copper transport: import*

1.3.3.1 *Divalent metal transporter 1*

Divalent metal transporter 1 (DMT1) also known as Natural resistance-associated macrophage proteins (Nramp) or Divalent cation transporter 1 (DCT1) is thought to be the primary protein responsible for iron absorption in the small intestine (Figure 1.2)(Gunshin, Mackenzie et al. 1997; Arredondo, Munoz et al. 2003; Arredondo, Cambiazo et al. 2004). This transporter has relatively little specificity for the type of metal ion it transports as it is capable of transporting a number of divalent cations including Fe^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+} and Cd^{2+}(Gunshin, Mackenzie et al. 1997). Because this transporter has been shown to transport Cu^{2+}, it has become a candidate protein for the transport of copper into other cells besides intestinal cells.

DMT1 is a protein containing 12 trans-membrane segments and 561 amino acid residues. The protein is widely expressed as ascertained by mRNA levels with highest mRNA levels in the intestine, kidney and some regions of the brain (Gunshin, Mackenzie et al. 1997). Transport of iron by DMT1 has been demonstrated in an oocyte expression system and been shown to be electrogenic. It was shown to co-transport H^+ ions as well as to act as a uniporter of H^+ ions (Gunshin, Mackenzie et al. 1997) and with the transport of Fe^{+2} it generates a positive inward current(Tandy, Williams et al. 2000). A single change at residue G185 mutated to an arginine causes defective iron transport into the

intestine of the microcytic mouse and the Belgrade rat (Fleming, Trenor et al. 1997; Fleming, Romano et al. 1998).

DMT1 expression appears to be tightly regulated by Fe^{+2} requirements in cells. This regulation is apparent at both mRNA (Canonne-Hergaux, Gruenheid et al. 1999) levels as well as protein levels (Fleming, Migas et al. 1999). Most of the work that suggests DMT1 is a relevant transporter has been carried out in Caco-2 cells. Copper uptake by Caco-2 cells has been shown to occur suggesting DMT1 may provide a pathway for copper to enter cells. Using competition experiments, researchers have shown that uptake of copper and iron is related in Caco-2 cells. At high extracellular iron levels, DMT1 protein expression was shown to be reduced and copper uptake in these same cells was reduced as well. Tennant et al found that at high extra-cellular copper levels, both DMT1 mRNA and protein levels were reduced (Tennant, Stansfield et al. 2002). Other have shown that copper uptake is reduced by 50% in Caco-2 cells when transfected with antisense oligonucleotides targeted at DMT1 (Arredondo, Munoz et al. 2003). This work suggests that in Caco-2 cells, DMT1 can transport copper across the plasma membrane. Recent reports suggests that DMT1 levels may be regulated by copper concentrations (Arredondo, Cambiazo et al. 2004). In lower copper concentrations, DMT1 expression increases and at higher copper concentrations DMT1 protein levels decrease. Based on transport studies reported by Arredondo et al, DMT1 may have an apparent affinity for copper in the low μM copper range (Km ~1 μM) (Arredondo, Cambiazo et al. 2004).

1.3.3.2 *Copper transporter 1*

The second set of proteins known to allow uptake of copper into cells is the copper transporter 1 (Ctr) family of proteins. This family of proteins has been shown to exist in a number of species and many different cell lines. Ctr1 is the focus of this thesis and will be described in detail below.

At the cellular level, copper is transported at the plasma membrane and distributed to cellular proteins and compartments for the incorporation of copper into copper –dependent proteins. The single cell organism, S. cervisiae, has two known high affinity copper transporters encoded by Ctr1 and Ctr3 (Dancis, Yuan et al. 1994; Knight, Labbe et al. 1996). Yeast cells lacking Ctr1 and Ctr3 exhibit striking defects in copper and iron uptake, mitochondrial respiration and Cu/Zn superoxide dismutase activity (Dancis, Yuan et al. 1994; Knight, Labbe et al. 1996; Labbe, Pena et al. 1999; Zhou and Thiele 2001).

Ctr1 in yeast was identified in 1994 as a consequence of a defect in iron absorption (Dancis, Yuan et al. 1994). Essentially a yeast strain defective in iron transport was found to have a defect in a gene that was determined to be a copper transporting protein, yCtr1. Fet3p, a plasma membrane iron oxidase requires copper as a cofactor. yCtr1p is responsible for the transport of copper into yeast and therefore allows copper to be incorporated into Fet3p. yCtr1 was found to be a 406 amino acid protein that contained two hydrophobic stretches of amino acids (153-177) and (242-281)(Dancis, Haile et al. 1994; Dancis, Yuan et al. 1994). Given the length of this second hydrophobic stretch it was postulated that this hydrophobic domain was actually two trans-membrane segments (Dancis, Yuan et al. 1994). The protein was localized to the plasma membrane of yeast using an epitope tag at the carboxyl terminal end of the

protein. The protein migrated on an SDS –PAGE gel as a protein band of 100 kDa, however the predicted size of yCtr1 is 47-49 kDa. It was shown using yeast strains that have temperature sensitive mutations for glycosylation that yCtr1 contains significant O-linked glycosylation (Dancis, Haile et al. 1994).

yCtr1 also contains multiple MXXM motifs in it amino-terminal domain as well as in its second trans-membrane domain. These motifs are thought to be important for copper interaction with the protein as it is transported across the membrane (Dancis, Yuan et al. 1994; Puig, Lee et al. 2002). Using yCtr1p expressed with either an HA or c-myc tag at the carboxyl terminal end of the expressed protein it was demonstrated by co-immunoprecipitation that yCtr1 likely formed at least dimers and possibly higher order oligomers (Dancis, Haile et al. 1994). This is reasonable as most transport proteins contain 6- 12 trans-membrane domains. yCtr1 was also shown to mediate radio-isotopic copper uptake in yeast cells demonstrating the yCtr1 is a functional copper transporter.

A second protein that is capable of transporting copper into yeast cells is yCtr3, first discovered in 1996 (Knight, Labbe et al. 1996). The CTR3 gene is interrupted in most laboratory yeast strains by the insertion of a transposable element in its promoter region. Ctr3p is protein of 241 amino acids and contains 11 cysteine residues in contrast to yCtr1 (Knight, Labbe et al. 1996). It is capable of high-affinity Cu uptake, and can restore Cu, Zn superoxide dismutase activity, ferrous iron transport, and respiratory defects in strains lacking yCtr1p (Knight, Labbe et al. 1996; Pena, Puig et al. 2000). Yeast strains must be defective in both of these transporters in order to show the copper deficiencies associated with lack of copper uptake. Although Ctr1p and Ctr3p can function independently in copper transport, the expression of both proteins provides

maximal copper uptake and growth rate under copper- limiting conditions. Ctr3 can assemble as a trimer at the plasma membrane similarly to yCtr1. This has been demonstrated through the use of the chemical crosslinker ethylene glycol bis(succinimidyl)succinate (Pena, Puig et al. 2000).

A difference between yCtr1 and yCtr3, other than their relative size, is the presence of multiple cysteine residues in yCtr3 and the relative lack of methionine residues (Figure 1.3). Significantly, four of these residues (C16, C48, C51 and C199) are important for copper incorporation into yeast (Pena, Puig et al. 2000). The exact role these residues plays is unknown. It has been postulated that they play a role in proper folding or dimerization of the subunits to assemble a functional unit (Pena, Puig et al. 2000).

Copper uptake is regulated both at transcriptional and post translational levels in S. cerevisiae. Transcription factors that regulate the expression of Cu homeostasis genes have been identified. In yeast, control occurs at the transcriptional level regulated by two copper sensing and copper modulated transcription factors: Mac1p (metal binding activator) and Ace1p (activator of CUP1 expression) (Furst, Hu et al. 1988; Jungmann, Reins et al. 1993). Mac1p is active in low Cu conditions and induces the expression of genes necessary for copper uptake. Copper ion-responsive elements exist in the promoters of both yCtr1 and yCtr3. Transcription of Ctr1 and Ctr3 genes is regulated through the action of the copper-sensing transcription factor Mac1p, and phosphorylation of Mac1p is likely linked to this transcriptional regulation. Mac1p regulates the expression of three genes necessary for the uptake of copper into yeast cells. Elevated copper levels inhibit Ctr1 and Ctr3 specifically while Fe and Cu modulate expression of

Fre1 (Jungmann, Reins et al. 1993; Dancis, Haile et al. 1994). This suggests that genes involved in copper uptake in yeast cells are regulated through the sensing of Cu ions in intra-cellular compartments. Mac1p was shown to have a copper sensing activation domain by the fusion of this activation domain (residues 42-417) to the Gal 4 DNA binding domain (residues 1-147). Extra cellular copper represses this construct. It was also shown that a fusion of $Mac1^{up1}$ ($Mac1^{up1}$ is mutant that exhibits copper hypersensitivity) with Gal4 DNA binding domain abolished this copper repression (Graden and Winge 1997). Therefore the activation of Mac1p for gene expression was separated from the repressive effects seen through its interaction with copper ions.

Others identified a common promoter element for the three genes, Fre1 Ctr1 and Ctr3. This sequence named CuRE (copper- response element) has the consensus sequence 5'-TTTG(T/G)C(A/G)-3' found in two copies in each of the promoters for these genes and was determined to be necessary for both copper repression and activation in copper-depleted cells (Labbe, Zhu et al. 1997). *In vivo* footprinting studies showed the CuRE sequences were occupied under copper depleted conditions and accessible to DNA modifying agents in copper replete cells. It was shown that a direct interaction between the CuRE and Mac1p occurred by using electrophoretic mobility shift assays (Yamaguchi-Iwai, Serpe et al. 1997).

Additional work on the regulation of Mac1p in response to copper levels in the cell demonstrated that the Mac1p is very stable at low Cu concentrations. However when intracellular Cu levels are high Mac1p undergoes a rapid degradation that is consistent with the reduction in Ctr3 mRNA levels as assayed by RNAse protection (Zhu, Labbe et al. 1998). It was also demonstrated that Mac1p is degraded. The carboxyl terminal

activation domain was shown to be involved in this copper regulated degradation process. Mutations in the two cysteine rich repeats were found to be responsible for the degradation patterns of Mac1p and activation of Ctr3 gene expression (Zhu, Labbe et al. 1998).

Jensen and Winge showed that Mac1p is a copper binding protein (binds 8 Cu ions) and that upon the binding of copper Mac1p undergoes a conformational change (Jensen and Winge 1998). This change allows an inter-molecular interaction between the N-terminal and C-terminal portions of the protein. This intra-molecular interaction inhibits both DNA binding and transactivation by Mac1p (Jensen and Winge 1998). These authors also showed that the $Mac1^{up1}$ protein was more stable than wild type in high copper concentrations. The $Mac1^{up1}$ also had only 4-5 copper ions bound suggesting that Mac1p is acting to measure intracellular copper levels. Jensen and Winge suggest a model for Mac1p function. Their model states that under copper depleted conditions there is no interaction between the N-and C- terminal portions of the protein, which allows the DNA binding domain to interact with DNA and the activation domain to be exposed to interact with general transcription factors within the mediator complex. Under copper replete conditions, Mac1p N- and C-terminal domains interact causing the loss of DNA binding activity.

Jensen et. al. later showed that the first 159 residues of Mac1p are the minimal DNA binding domain(Jensen, Posewitz et al. 1998). They also showed that both CuRE elements are necessary for efficient transcriptional activation (Jensen, Posewitz et al. 1998). They described the binding of two Zn ions to this N-terminal domain and suggest that the N-terminal 40 amino acids are a DNA binding Zn module. This type of module

is found in Ace1 and Amt1 (Jensen, Posewitz et al. 1998). They also show that two repeats of the CuRE sequence are not needed for DNA binding but two are needed for high level transcriptional activation *in vivo.*

It was discovered that the transcriptional activity of Mac1p was localized to amino acids 264-279, a cysteine rich motif in the middle portion of Mac1p. Additionally a helix motif at the C-terminus is likely to be involved in the protein–protein interactions of Mac1p with itself. Point mutations within this helix motif abrogated *in vivo* transcription activated by Mac1p (Serpe, Joshi et al. 1999).

Elevated levels of copper lead to the repression of Mac1p and the activation of Ace1p. Under high copper levels Ace1p induces the expression of genes encoding copper binding proteins namely Cup1 and Crs5 (two yeast metallothioneins) and the cytoplasmic Cu/Zn SOD1. These proteins protect against the toxic effects of copper by binding to copper (metallothioneins and SOD1) and by abrogating reactive oxygen species generated by copper redox chemistry (SOD1) (Furst, Hu et al. 1988; Gralla, Thiele et al. 1991; Culotta, Howard et al. 1994; Culotta, Joh et al. 1995). To date, no transcription factor regulating mammalian copper homeostatic genes has been identified.

Yeast copper uptake is also controlled at the post-translational level (Ooi, Rabinovich et al. 1996). yCtr1p undergoes Mac1-dependent degradation in response to high levels of copper ions, in the apparent absence of endocytosis. The exact mechanism of Mac1-dependent degradation is not known, however two possible mechanisms have been suggested (Yonkovich, McKenndry et al. 2002). The first mechanism suggested is that Mac1 activates a gene encoding a protease that specifically targets yCtr1. A second suggested mechanism is that Mac1 is actually responsible for this degradation by either

binding to yCtr1 and targeting yCtr1 for degradation or that Mac1 has protease capabilities itself (Yonkovich, McKenndry et al. 2002). Furthermore, some plasma membrane localized yCtr1p is internalized into an endosome-like structure upon addition of copper. yCtr3p does not undergo this turnover and endocytosis (Ooi, Rabinovich et al. 1996; Pena, Puig et al. 2000).

Human Ctr1 was cloned by complementation of the yeast high affinity copper uptake mutant deficient for Ctr1 and Ctr3 (Ctr1/3)(Zhou and Gitschier 1997). Based on hydropathy analysis, hCtr1 is similar in overall structure to the yeast copper transporters Ctr1 and Ctr3 (Figure 2.1). Besides complementing the growth defect on non-fermentable media associated with the Ctr1/3 mutant, the human Ctr1 gene also rescues iron transport and Cu/Zn superoxide dismutase defects in Ctr1/3 yeast. hCtr1-mediated ^{64}Cu transport is an energy-independent, saturable (K_m~3 μM) process and may be stimulated by extra-cellular acidic pH and possibly by high K^+ concentrations (Lee, Pena et al. 2002).

Based on sequence homology, a cDNA encoding an hCtr1-related protein, denoted hCtr2, was identified (Zhou and Gitschier 1997; Moller, Petersen et al. 2000). Both hCtr1 and hCtr2 are expressed in many human tissues examined and both genes are located at chromosome9q31/32. Expression of hCtr2 did not complement the respiratory deficiency of the yeast lacking high-affinity copper transporters, and did not alter the kinetics of ^{64}Cu uptake or export in fibroblast cells (Moller, Petersen et al. 2000). These observations suggest that hCtr2 is not likely to function as a high affinity copper transporter in a manner analogous to hCtr1.

Competition experiments support a hypothesis that hCtr1 is a specific metal transporter. Strong competition by Ag(I) in copper uptake experiments suggests that

Ag(I), which is isoelectric to Cu(I), can be transported by hCtr1 and that reduced monovalent copper is the preferred substrate for the hCtr1 transporter since divalent metals (Zn, Fe, Mg) do not compete with copper for transport(Lee, Pena et al. 2002). Consistent with this observation, ascorbate treatment to reduce Cu(II) to Cu(I) may enhance copper uptake, and studies in bakers yeast strongly suggest a role for the FRE1 and FRE7 metallo-reductases as physiological enzymes in the reduction of copper before cellular import (Hassett and Kosman 1995; Georgatsou, Mavrogiannis et al. 1997; Martins, Jensen et al. 1998).

Targeted gene deletion of the mCtr1 gene results in embryonic lethality in homozygous mutant embryos demonstrating the critical function of Ctr1 in embryonic development (Kuo, Zhou et al. 2001; Lee, Prohaska et al. 2001). Mice heterozygous for Ctr1 display tissue specific defects in copper accumulation, but are phenotypically normal. Analysis of tissue copper-content reveals that the brain and spleen copper levels were reduced approximately 50% in $Ctr1^{+/-}$ mice, however gut, liver and kidney copper levels were not different from wild-type control mice (Kuo, Zhou et al. 2001; Lee, Prohaska et al. 2001). Consistent with these findings, brain copper-enzyme activity was also reduced in $Ctr1^{+/-}$ mice. The Ctr1 knockout experiments in mice demonstrate that Ctr1 activity is rate limiting for copper uptake in several organs and demonstrated the lack of a functionally redundant high affinity copper transport activity in mammals.

This essential requirement for hCtr1 in mice underscores the ubiquitous presence of hCtr1 in various tissues and cell types. Many tissues and cell types investigated contain hCtr1 proteins or mRNA (Zhou and Gitschier 1997; Lee, Prohaska et al. 2000; Klomp, Tops et al. 2002). The specific level of protein expression and the localization of

hCtr1 protein in the cell has become the focus of a number of studies (Klomp, Tops et al. 2002; Petris, Smith et al. 2003). These investigators look at either endogenously expressed hCtr1 or transfected epitope-tagged hCtr1 to ascertain the cellular localization of hCtr1. In many cell types (Caco-2, HEK 293 and CHO), hCtr1 is localized to the plasma membrane as expected for mediating the transport of copper into cells (Klomp, Tops et al. 2002; Lee, Pena et al. 2002; Petris, Smith et al. 2003). However in other cell types (HeLa, A549, H441 and BeWo), hCtr1 is primarily found in an intracellular unspecified location (Klomp, Tops et al. 2002). This has led to the investigation of whether hCtr1 is regulated in a copper-specific manner. Petris et al have suggested that hCtr1 is regulated in a copper specific way (Petris, Smith et al. 2003), however this has been disputed and is a focus of later chapters in this thesis.

The amino acid sequence of the human high affinity transporter Ctr1 contains three methionine-rich domains, called Mets-domains; two in the N-terminal part of the protein and one in the second putative trans-membrane domain, whereas yCtr1p contains eight Mets-domains (figure 1.4 and 2.1). In addition, two histidine-rich domains are present in the N-terminal part of hCtr1. The first Mets-domain is not conserved among species and mutations in this domain have little effect on copper transport activity, further suggesting that this domain is not essential for Ctr1 function. Mutations in the two histidine-rich domains in the N-terminus of hCtr1 also do not influence copper transport activity (Puig, Lee et al. 2002). However, mutations at positions M43 and M45 in the second methionine rich domain are unable to complement the growth defect of the yeast high affinity copper uptake mutant, Ctr1/3, and only modestly stimulate ^{64}Cu uptake in human cells, compared to the wild-type hCtr1 allele. Two other methionines in the

predicted second trans-membrane domain of hCtr1 protein, M150 and M154, are also conserved among Ctr1 proteins in different species (Figure 1.4), and mutational analysis demonstrated that both methionine residues are important for the ability of hCtr1 to complement the growth defect of yeast cells lacking Ctr1/3 (Puig, Lee et al. 2002). Furthermore, expression of these mutant alleles reduces copper uptake in HEK 293 cells expressing wild-type hCtr1, suggesting that multimers are formed between mutant and wild-type alleles of hCtr1. Although these data indicate the significance of some amino acids in Ctr1 mediated copper transport activity, further analysis of the biochemical characteristics and expression pattern of Ctr1 protein is necessary to understand Ctr1 function.

1.4 Questions addressed in this dissertation.

Many unresolved questions remain concerning hCtr1 and its role as a copper transporter. Some of these include how copper is transported through the membrane? Does copper bind to specific residues in hCtr1 as it passes through the membrane? What residues are important for the proper folding of hCtr1 and are there structural domains that impact transport of copper across the membrane? Is hCtr1 functional at the plasma membrane? Is hCtr1 post translationally modified? Is hCtr1 regulated transcriptionally or post translationally? If so, is the regulation copper dependent?

The remainder of this thesis focuses on several of these unanswered questions regarding structure, function and regulation of hCtr1. Specifically, Chapter 2 focuses on basic protein structural questions concerning topology of the protein, and identification of residues that are post translationally modified or are important for structural stability. Chapter 3 continues the investigation of copper transport by focusing on particular

domains and residues that may play a role in the transport mechanism. Chapter 4 investigates the mechanism of copper transport by asking questions about the regulation of endogenous hCtr1. Specifically, does hCtr1 transport copper at the plasma membrane and is there any significant regulation of this process. Chapter 5 continues an investigation of the role of regulation as it pertains to hCtr1 and focuses on whether a significant copper-dependent regulation exists for hCtr1 as seen with yCtr1 or is hCtr1 more similar to yCtr3 in certain aspects of its regulation.

Table 1.1 Copper containing proteins

This table consists of a partial list of proteins that require copper as a cofactor. Inadequate copper binding to some proteins can lead to disease pathology due to lack of function. Alternatively, many cellular processes, in the presence of too much copper can have altered function causing hyper-activity and toxicity.

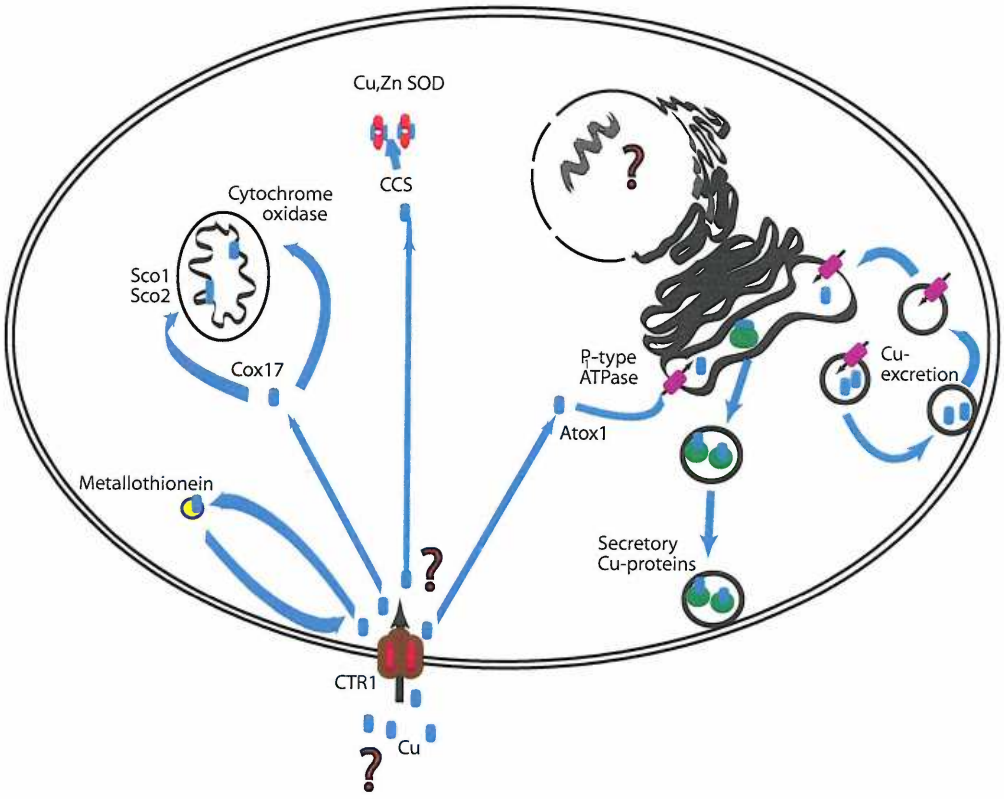
Table 1.1

Short list of Copper dependent proteins

Enzyme	Function
Angiogenin	Induction of blood vessel formation
β–amyloid precursor protein	unknown
Blood clotting factors V & VIII	Blood clotting
Ceruloplasmin	Iron homeostasis
Cu/Zn superoxide dismutase	Antioxidant defense
Cytochromme C oxidase	Mitochondrial respiration
Dopamine b-hydroxylase	Neurotransmitter synthesis
Hephaestin	Intestinal iron efflux
Lysyl oxidase	Collagen crosslinking
Metallothionein	Cu sequestration
Monophenol monooxygenase	Melanin synthesis
Peptidylglycine a-amidating monooxygenase	Neuropeptide and peptide hormone processing
Tyrosinase	Melanin synthesis

Figure 1.1 Mammalian Cellular Cu Metabolism and trafficking. Many components of the copper metabolism pathways have been identified in mammalian cell. This figure summarizes the location and role played by these copper homeostatic proteins. Copper enters via Ctr1 and is picked up by cytoplasmic chaperones to be distributed to various cellular locations, including the mitochondria (Cox 17), cytoplasm (CCS) and trans-golgi network (Atox 1) or can be sequestered by metallothionein. Copper is incorporated into target proteins in the mitochondria, or cytoplasmic proteins such as SOD. A major pathway for the delivery of copper to secreted proteins or membrane proteins is through an Atox1- P1-ATPase interaction. The P_1-ATPases then transport copper into the TGN or excrete excess copper out of the cell.

Figure 1.1



Mammalian Cellular Cu Metabolism and Trafficking.

Figure 1.2 Copper dietary intake, delivery to and processing by the liver.

Ingested copper is absorbed by the enterocytes of the small bowel. Copper is transported across the apical membranes by two transporters, Ctr1 and DMT1. Copper is then excreted at the basolateral membrane by the Menkes protein for delivery to the liver. After processing and excretion from the liver by the Wilsons disease protein, copper is carried to the rest of the tissues in the body by small molecular weight peptides or more recently thought to be carried to other tissue by ceruloplasm.

Figure 1.2

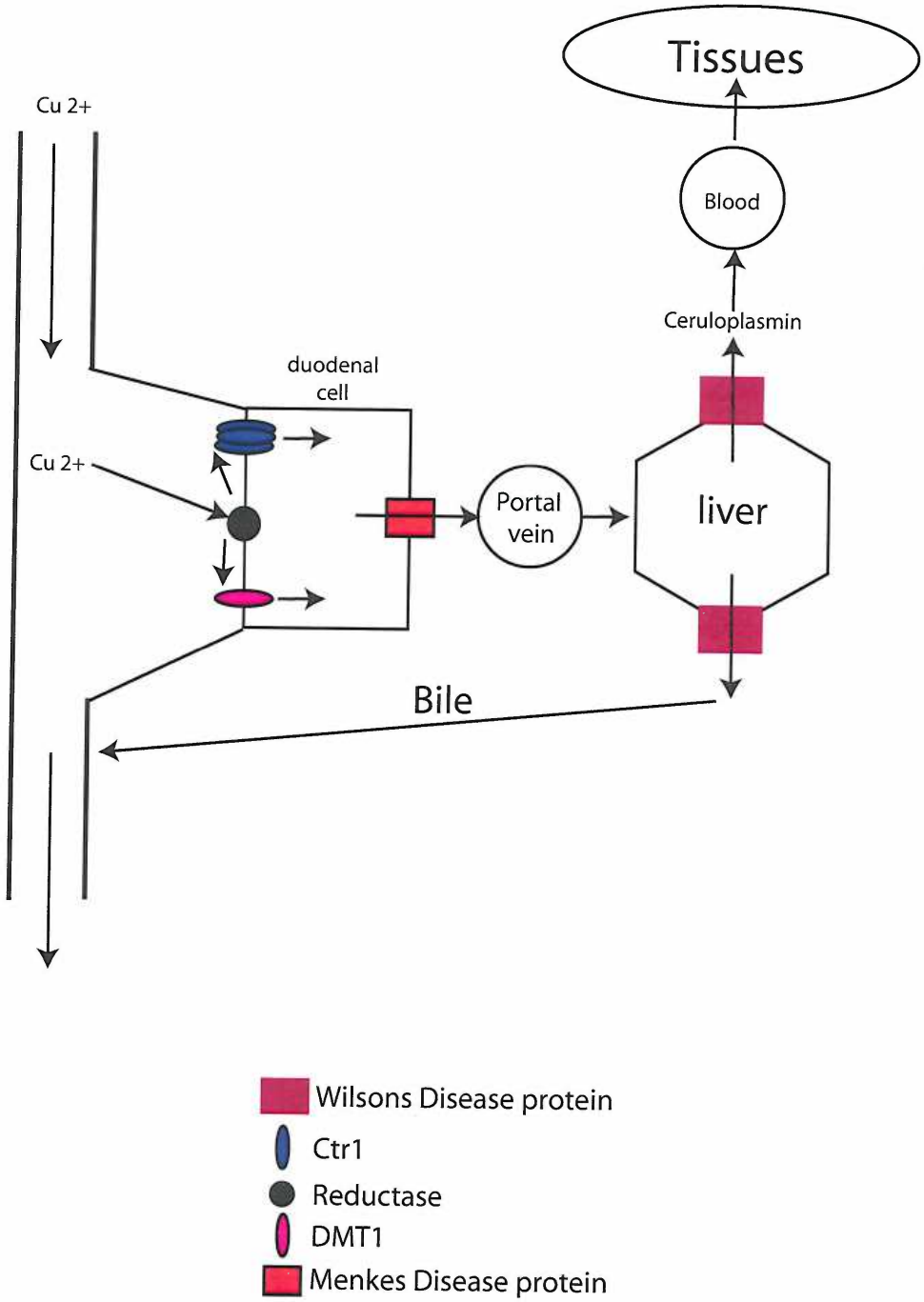
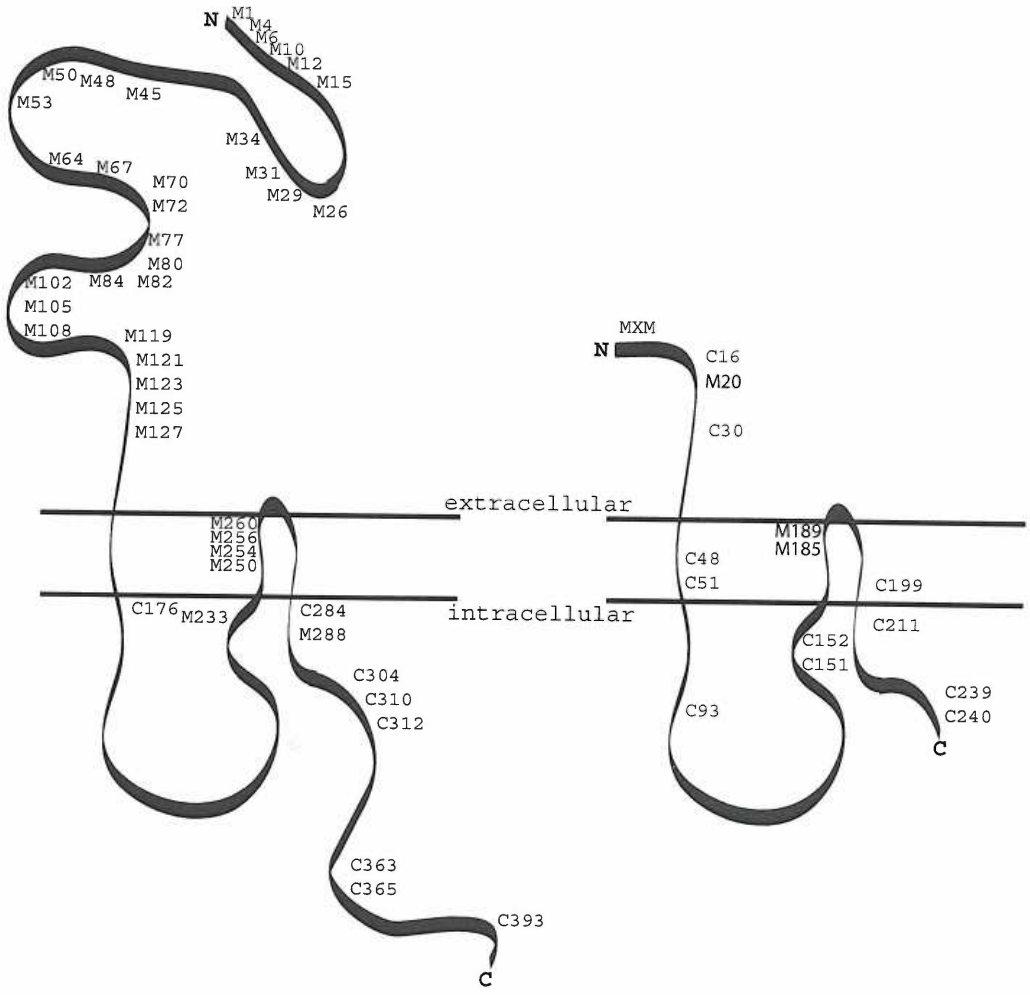

Tissues
Cu 2+
Blood
Ceruloplasmin
duodenal
cell
Cu 2+
Portal
vein
liver
Bile
Wilsons Disease protein
Ctr1
Reductase
DMT1
Menkes Disease protein

Figure 1.3 Schematic representation of the location of copper coordinating residues in yCtr1 and yCtr3. yCtr1 is relatively rich in Methionine residues while yCtr3 has numerous cysteine residues. Both are found in copper binding motifs and are target residues thought to bind copper during the transport mechanism. The figure also represents the relative size differences between the two yeast proteins.

Figure 1.3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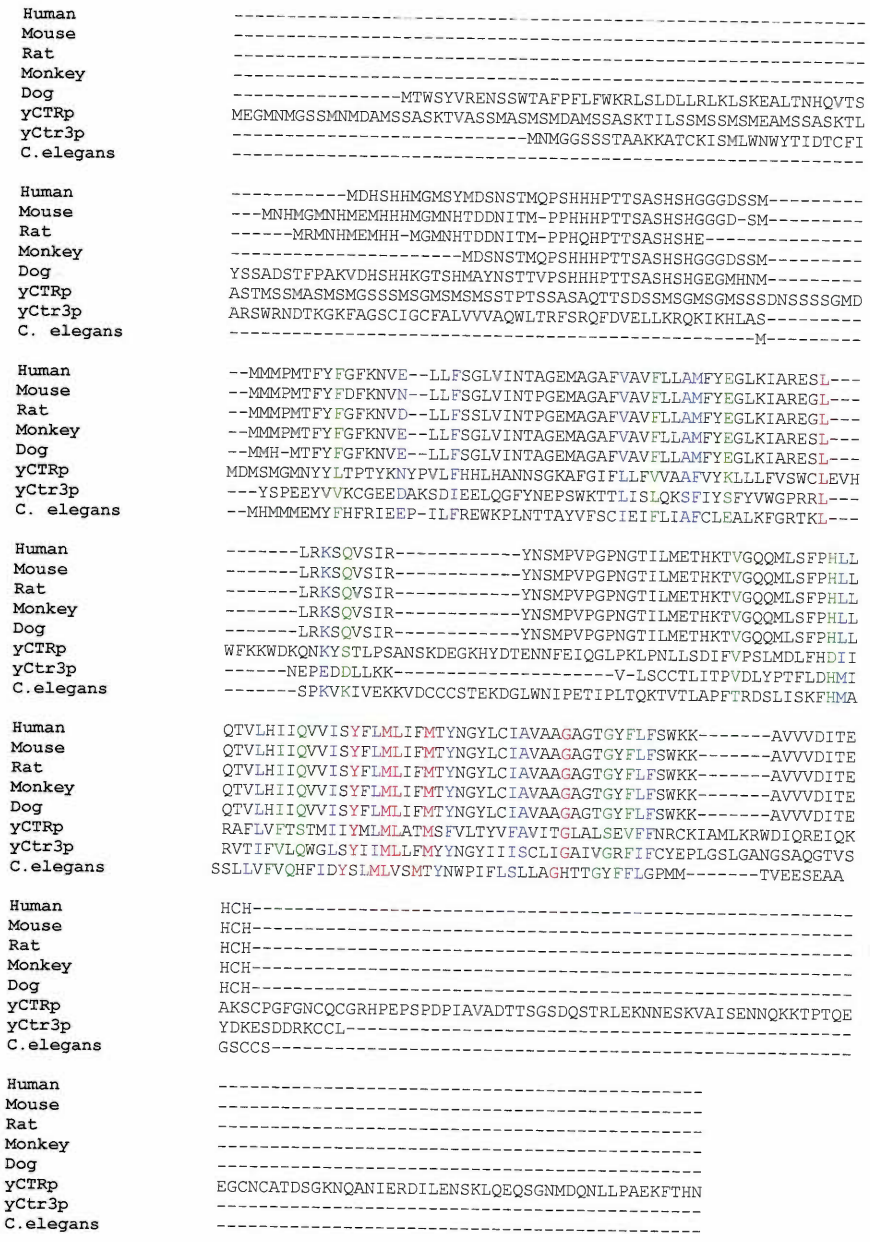
M121
M123
M125
M127
MXM
N
C16
M20
C30
extracellular
M260
M256
M254
M250
M189
M185
C48
C51
C199
C176
M233
C284
M288
intracellular
C211
C152
C151
C304
C310
C312
C93
C239
C240
C
C363
C365
C393
C

Figure 1.4 Ctr1 protein alignments. This figure contains protein alignments of several mammalian Ctr1 proteins with yeast and C.elegans Ctr proteins. The alignments show the relative lack of amino acid identity. This lack of identity has led some to postulate that structural considerations play a more important role in transport rather than actual binding of copper by specific residues.

Figure 1.4


Human
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C.elegans
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Chapter 2

Molecular Characterization of hCtr1, the Human Copper Uptake Protein.

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2.1 ABSTRACT

We have expressed hCtr1, the human Cu transporter, in *sf9* cells using a baculovirus-mediated expression system and observe greatly enhanced Cu uptake. Western blots show the protein is delivered to the plasma membrane, where it mediates saturable Cu uptake with a K_m of ~ 3.5μM. We also expressed functional transporters where the N-linked glycosylation sites are substituted and provide evidence for the extracellular location of the amino-terminus. Accessibility of amino-terminal FLAG epitope to Ab prior to permeabilization and of carboxy-terminal FLAG only after permeabilization confirms the extracellular location of the amino-terminus and establishes the intracellular location of the carboxy-terminus. Tryptic digestion of hCtr1 occurs within the cytoplasmic loop and generates a 10 kDa carboxy-terminal peptide; cleavage is prevented by the presence of Cu. hCtr1 mutants where C161 and C189, the two native cysteines are replaced with serines also mediate Cu uptake, indicating that neither cysteine residue is essential for transport. However, the mutants provide evidence that these residues may stabilize hCtr1 oligomerization. Western blots of hCtr1 in *sf9* cells show expression levels 100-fold higher than in mammalian (HepG2) cells. The high level of functional expression and the low level of endogenous Cu uptake will enable future structure-function analysis of this important protein.

2.2 INTRODUCTION

It has been estimated that about one third of all known proteins contain metal co-factors and the majority of these function as essential metalloenzymes. Cu is an essential micronutrient in humans, and it is required for cellular respiration, iron homeostasis, pigment formation, neurotransmitter biosynthesis, peptide biogenesis, connective tissue production and antioxidant defense (Culotta 2001). Recent studies in microorganisms and the identification and characterization of the molecular basis of several genetic diseases of Cu metabolism in humans have provided a partial list of the components involved in regulating and controlling intracellular Cu metabolism (DiDonato and Sarkar 1997; Valentine and Gralla 1997; Askwith and Kaplan 1998; Schaefer and Gitlin 1999; Harrison, Jones et al. 2000; Bartnikas and Gitlin 2001). The transport of Cu into and out of cells has also received increased attention. Mutations in either of two human genes encoding Cu-transporting P-type ATPases that are localized in the secretory pathway result in Menkes Disease (ATP7A) and Wilsons Disease (ATP7B), which cause intestinal Cu absorption defects or maldistribution of hepatic Cu respectively (DiDonato and Sarkar 1997; Schaefer, Roelofsen et al. 1999). The two P-type ATPases are clearly essential for the controlled removal of Cu from cells (Bull, Thomas et al. 1993; Chelly, Tumer et al. 1993; Tanzi, Petrukhin et al. 1993; Vulpe, Levinson et al. 1993; Yamaguchi, Heiny et al. 1993). The importance of these proteins was established in1993, however, at that time the mechanism of Cu uptake into mammalian cells was entirely unknown.

An important series of studies in the yeast *S. Cerevisiae*, first appearing in 1994, provided the basis for our current ideas about the major Cu entry mechanisms (Dancis,

Haile et al. 1994; Dancis, Yuan et al. 1994). It was shown that a gene, CTR1, encoded a multi-spanning plasma membrane protein that was specifically required for high affinity Cu transport into yeast. The CTR1 gene product is a protein composed of 406 amino acid residues. A second high affinity Cu uptake protein, encoded by the CTR3 gene was subsequently identified. The two gene products are functionally redundant and both contain three putative transmembrane segments, however, they are structurally quite different (Pena, Puig et al. 2000). Ctr3 is composed of 241 amino acid residues, and it lacks the MXXMXM motifs that are abundant in Ctr1. Ctr3 is relatively rich in cys residues, containing 11 cys residues in its sequence, although apparently only 4 of these residues may be important for function (Pena, Puig et al. 2000). Ctr1 is highly glycosylated (Dancis, Haile et al. 1994) and has eight repeats of the MXXMXM motifs. It was speculated that the glycosylation had some functional significance (Dancis, Haile et al. 1994). The Mets motifs, which are regarded as potential metal-binding sites, are located in the amino-terminal tail that is thought to be in the predicted extracellular domain. The presence of these multiple sulfur-containing amino acid residues suggested a possible role in Cu coordination. It should be borne in mind that the hydropathy analysis of these transporters suggests three transmembrane segments (see Figure 1) and thus the amino- and carboxy-terminals are on opposite sides of the membrane. The reason that the amino-terminus was placed on the outside in the initial models was that the Mets domains were in the amino-terminus and since the proteins were Cu uptake systems, Cu binding was assumed to be the first step in the process. Obviously two alternatives exist, with the amino-terminus at the extracellular surface and the carboxy-terminus in the cytosol, as assumed, or vice versa.

In 1997 Zhou and Gitschier identified the first human gene for Cu uptake (Zhou and Gitschier 1997). This protein, hCtr1, was identified by complementation of a Ctr1 growth defect in yeast on non-fermentable media and also rescued iron transport and Cu/Zn SOD defects. The gene was proposed to encode a high affinity Cu uptake process and was found to be 29% identical at the primary structure level with Ctr1 from yeast. The human protein was, however, substantially smaller than the yeast protein being composed of 190 amino acids as compared with 406 amino acid residues (see Figure 1). hCtr1 is on the one hand a smaller protein, like yCtr3 (241 residues), but has Mets motifs like yCtr1. The amino-terminal domain of hCtr1 has been described as having two Mets motifs, however, the first of these is MXMXXM, rather than the usual MXXMXM, and the second is unusually methionine-rich in having the sequence MMMMXM. hCtr1 also has a methionine-rich sequence at the extracellular boundary of the second putative transmembrane segment that may be important for function. hCtr1, in contrast to Ctr3, is a very cys-poor protein having only two cys residue, C161 and C189, in its sequence. The murine homolog of hCtr1, termed mCtr1, was isolated by Lee *et al.* three years later and was shown to have 92% protein sequence identity with hCtr1 (Lee, Prohaska et al. 2000). Mouse Ctr1 (mCtr1) in fact has only 4 residues that differ from hCtr1 beyond the first 37 residues. Mouse Ctr1 complemented yeast strains that were defective in high affinity Cu uptake. The mammalian Ctr1RNA was expressed in all tissues examined, with higher levels in kidney, liver and lower levels in brain and spleen (Lee, Prohaska et al. 2000). Recent studies have established that expression of mammalian Ctr1 is essential for embryonic development (Kuo, Zhou et al. 2001; Lee, Prohaska et al. 2001). It was shown that homozygous knock-outs resulted in embryonic lethality, while heterozygous

animals exhibited tissue-specific defects in Cu accumulation and in the activities of Cu-dependent enzymes. In a publication that appeared during the preparation of this article (Lee, Pena et al. 2002), an initial characterization of hCtr1 in mammalian cells has been reported. The over-expression of hCtr1 in HEK 293 cells was described and evaluated by measuring ^{64}Cu uptake. These authors showed time-dependent, saturable Cu uptake with an apparent affinity of about 3μM.

We now describe the functional expression of hCtr1 in *sf9* cells following infection with baculovirus particles containing the cDNA for hCtr1. Heterologously expressed hCtr1 is delivered to the plasma membrane where it mediates saturable Cu uptake. Utilizing mutagenic analysis of expressed hCtr1 molecules we show that neither of the two Cys residues, C161 or C189 play a role in Cu uptake and that glycosylation of hCtr1 is not required for function. By combining these results with experiments on proteolytic susceptibility, and Ab accessibility we provide strong evidence for a three transmembrane segment model with an extracellular amino-terminus and an intracellular carboxy-terminus. The functional heterologous expression of hCtr1 in insect cells will enable a detailed investigation of the mechanism of this important transporter.

2.3 METHODS

2.3.1 *Cloning of hCtr1*

The cDNA for hCtr1 was obtained from the laboratory of Dr. Jane Gitschier, UCSF. hCTR1 was PCR-cloned into pFastBacDual vector (pFBD; Invitrogen, Carlsbad Ca.) as an EcoRI – HindIII fragment. Additionally, hCTR1 was PCR-amplified to insert a FLAG epitope (N-DYKDDDDK-C) at the N-terminus of hCtr1 as well as a construct containing a FLAG epitope at the C-terminus of hCtr1. Mutagenic primers were designed to introduce single amino acid substitutions at amino acid N15Q, N112Q, C161S, C189S (Table 1). Mutant cDNA's of hCtr1 were cloned into pFBD vector (Invitrogen, Carlsbad CA) as EcoRI-HindIII fragments. The cloned fragments were used to generate recombinant baculovirus following manufacturer's protocols (Invitrogen, Carlsbad CA).

2.3.2 *Transfection and Expression of hCTR1 in Insect Cells*

Briefly, cloned mutant cDNA were allowed to transpose into recombinant bacmids within DH10 Bac cells (Invitrogen, Carlsbad CA). Colonies containing hCTR1 mutant bacmids were then used to produce virus particles by transfecting *sf9* insect cells with the recombinant bacmid DNA. The resulting virus stocks were amplified twice and then used to express protein using *sf9* cells (Hu, Eisses et al. 2000). *sf9* cells were infected at a cell density of 1.0×10^6 cells/ml in spinner flasks. Cells were collected and disrupted via Dounce homogenization to produce unfractionated or fractionated membranes (Hu, Eisses et al. 2000; Hu and Kaplan 2000) containing over-expressed hCtr1, followed by Western analysis.

2.3.3 *Western Analysis*

Western analysis was performed using 50 μg of membranes loaded on a 12% Laemmli gel. β-mercaptoethanol (.2% v/v) was added to samples 15 min prior to electrophoresis at room temperature unless indicated. The gel was electro-blotted to nitrocellulose membrane in 10 mM CAPS (pH 11) and blocked using phosphate buffered saline (PBS; 137mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_20$, 1.4 mM KH_2PO_4, pH 7.4) containing 5% milk. After blocking, the membranes were incubated with a rabbit anti-C-terminal hCtr1 antibody (n-WKKAVVVDITEHCH-c; 1:5000 dilution) and secondary detection was carried out using a goat anti-rabbit antibody (1:5000).

2.3.4 *Copper Transport by hCTR1*

Copper uptake experiments were performed following previously published protocols (Gatto, McLoud et al. 2001) for Rb uptake. Briefly, 12 well plates were seeded with *sf9* cells (1.0×10^6 cells /well). Virus was applied to each well of attached cells and allowed to incubate at 27°C. 72 hours post-infection, cell media were replaced with transport buffer (150mM NaCl, 2.5 mM $MgCl_2$, 25 mM Hepes, pH 7.4) and incubated at 27°C for 30 min. Cu transport was carried out using either ^{64}Cu (MIR Radiological Sciences, NIH Resource grant 1R24CA 86307, Washington University Medical School) or ^{67}Cu (Brookhaven National Laboratory) at specified $CuCl_2$ concentrations (10 μM $CuCl_2$ – time course experiments; .5, 1.0, 2.0, 5.0, 10.0, and 20.0 μM $CuCl_2$ –Cu dependence experiments). hCTR1-expressing cells were incubated for different times and then washed 3 times with ice-cold incubation buffer containing 10 mM EDTA. Cells were lysed using 0.1N NaOH and aliquots were counted using a γ -counter (Packard Cobra II) and also used to determine protein concentrations. Data were fit using the

Michaelis-Menten equation. The levels of expressed hCtr1 mutant protein were monitored by Western analysis using a C-terminal hCtr1 antibody as described above.

2.3.5 *PNGase F Treatment of hCTR1*

PNGase F treatment was performed on unfractionated membranes isolated from *sf9* cells expressing either N15Q hCtr1 or N112Q hCtr1. Briefly, 30 μg of membranes from each mutant sample were treated with 0.1% SDS/50mM β-mercaptoethanol for 15 min. at room temperature. NP-40 was added to a final concentration of 0.75 %. PNGaseF (2.5 mU, Glyko, Novato CA) was added and the mixture was incubated at 37°C for 8 hrs. Samples were run on a 12% Laemmli gel and analyzed by Western blot analysis. Antibodies raised in rabbits against the carboxy- terminus of hCTR1 were used in Western analysis following the protocol described above.

2.3.6 *Flag Antibody labeling of N-terminal and C-terminal tagged hCTR1*

N-terminal and C-terminal FLAG epitope-tagged hCTR1 (NT-FLAG or CT-FLAG) expressed in *sf9* cells were used to probe the topology of hCTR1. Intact *sf9* cells were surface-labeled using a mouse monoclonal anti-FLAG antibody (Anti-FLAG M2 Monoclonal antibody, Sigma, St. Louis, Mo.). Briefly, *sf9* cells were infected with baculovirus particles containing either a NT-Flag or CT-Flag construct and allowed to incubate for 72 hours. The cells were pelleted at 500 x g and resuspended in PBS. One half of the cells were used to prepare membranes for antibody labeling (membranes-M) and the other half were used to label whole cells with antibody (intact cells-IC). Antibody labeling was carried out for 2 hours at 4°C for both samples (M and IC, NT-Flag and CT-Flag hCTR1). The cell suspension was washed four times with PBS

containing 1 mg/ml BSA, for 15 minutes at 4°C. The IC samples were then used to prepare total membranes. Unfractionated membranes (500 μg) were solublized in incubation buffer (150mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$, 25 mM Hepes, pH 7.4) containing 2% n-Dodecyl-β-D-maltoside (DDM buffer; Calbiochem, San Diego, Ca.). Following solubilization, the samples were diluted to 0.2% DDM buffer and protein G Sepharose (Pharmacia Biotech, Piscataway, N.J.) was added for an overnight incubation at 4°C. The protein G Sepharose was pelleted at 500 x g, followed by three 1 ml washes with 0.2% DDM buffer. Protein G Sepharose was resuspended in 2X sample buffer (equal volumes of 8M urea, 10% SDS, and 125 mM Tris buffer, pH 6.8, no β-ME added) and run on a 12% Laemmli gel, followed by Western analysis (see above) using a rabbit anti-FLAG antibody (1:5000 dilution; ABCAM, Cambridge, UK).

2.3.7 *Trypsin Digestion of hCTR1*

Trypsin digestion of C189S hCtr1 was carried out on unfractionated membranes (TM) from *sf9* infected insect cells. TM samples (100μg) of C189S were incubated with 40 μg of TCPK treated Trypsin in incubation buffer for 1 hour at 27°C. 80 μg of Soybean Trypsin Inhibitor was added for 30 minutes and then samples were washed twice with incubation buffer to remove any remaining trypsin. TM samples (50 μg) were loaded on a 12% Laemmli gel for subsequent Western analysis using an anti-C-terminal hCtr1 antibody for detection.

2.4 RESULTS

2.4.1 *Functional expression of hCtr1*

We have recently optimized the baculovirus infection of *sf9* cells for the heterologous expression of the renal Na, K-ATPase (Hu, Eisses et al. 2000; Hu and Kaplan 2000; Gatto, McLoud et al. 2001) and for ATP7B, the Wilson Disease protein (Tsivkovskii, Eisses et al. 2002). In the present work we have infected *sf9* cells with a baculovirus construct containing the cDNA for hCtr1 (provided by Dr Jane Gitschier, UCSF) and after three days of infection, the cell membranes were separated using sucrose gradient sedimentation to yield endoplasmic reticulum, golgi apparatus and plasma membrane fractions (Hu and Kaplan 2000). Western blot analysis using an Ab raised against a peptide derived from a sequence in the carboxy-terminal of the transporter (see Experimental Methods) showed that the infected cells synthesized hCtr1 and following synthesis, hCtr1 was delivered to the plasma membrane (Figure 2.2A). Figure 2A shows the major bands of hCtr1, in each membrane fraction, which corresponds to monomeric protein. However multimerization (i.e. bands at higher mass) is seen in all fractions and is discussed further below (see role of cysteine mutants and Figure 2.6). When we optimized this expression system for the Na pump, we developed a ^{86}Rb isotopic uptake assay for the transport properties of the plasma membrane-incorporated expressed protein (Gatto, McLoud et al. 2001). In the present studies, we modified this protocol to measure the rate of Cu uptake into uninfected *sf9* cells and cells infected with baculovirus particles that contained hCtr1 cDNA. The results of such experiments are shown in Figure 2.3. It is clear that infected cells show a greatly enhanced rate of Cu uptake compared with uninfected cells (Figure 2.3A). If the studies are carried out at varied levels of

extracellular Cu two points are immediately apparent. First, infected cells expressing hCtr1 show an enhanced Cu uptake rate that saturates with increasing Cu concentrations, and secondly, uninfected cells show only very low rates of uptake (Figure 2.3B). In fact, in the hCtr1-expressing cells, the maximal rate of Cu uptake is some twenty- to thirty-fold higher than in the uninfected cells. The Cu concentration at half-maximal uptake rates is about 3-4 μM. This value agrees quite well with that recently reported in studies where hCtr1 was expressed in HEK 293 cells (Lee, Pena et al. 2002).

Since we expressed hCtr1 in *sf9* cells in the hope that its expression would be high so that we could carry out a characterization of its properties, we thought it would be interesting to compare the expression level we obtain in insect cells to that endogenously expressed in a mammalian cell line. In Figure 2.2B, we show a comparison of the Western blots of heterologously expressed hCtr1 in *sf9* cell membranes and the endogenously expressed hCtr1 in HepG2 cells. Clearly expression in the insect cell system is greater, and we estimate that the expression in the insect cells is 100-fold higher than in the HepG2 cells. In Figure 2.2B, it is apparent that the mobility of hCtr1 in HepG2 cells (35 kDa) differs from that of monomeric hCtr1 (28 kDa) expressed in *sf9* cells. We believe that this is due to the more extensive glycosylation of membrane proteins in mammalian cells compared with insect cells. The band at 46 kDa, in the insect cells is due to stable oligomerization of wild-type protein (see below and Figure 2.6 for discussion). We have not observed multimerization of endogenous hCtr1 in HepG2 cells. The significance of this difference is currently under investigation.

2.4.2 *Membrane topology of hCtr1*

Hydropathy analysis of the sequence of hCtr1 strongly suggests the presence of three transmembrane segments (see Figure 2.1). It has been assumed that the amino-terminus is extracellular and the carboxy-terminus is located in the cytosol. We noticed that the hydropathy analysis placed the two consensus sequences for N-linked glycosylation sites, N15 and N112 on opposite sides of the first putative transmembrane segment (Figure 2.1B). There are two possible membrane topologies if hCtr1 has three transmembrane segments (see Figure 2.4A). We then engineered and expressed two substitution mutants N15Q and N112Q of hCtr1, reasoning that if the hydropathy analysis established the correct number of transmembrane segments with the amino-terminus at the extracellular surface, then N15 would be exposed and glycosylated while N112Q would not be glycosylated as it would be in the cytoplasm. If we were going to use these substitution mutants to address the membrane orientation of hCtr1, it was first necessary to establish that such substitution mutants were functional. In Figure 2.4B, we show that the apparent affinity for Cu uptake is largely unaffected by these substitutions. These data also indicate that the absence or presence of glycosylation have no obvious effect on the transport functions of hCtr1. The data, shown in Figure 2.4C, support our speculations on the probable membrane orientation and glycosylation status of hCtr1. Expression of the N15Q hCtr1 produces a protein that has an apparent mass of 23 kDa, which is unaltered by treatment with PNGase F. Expression of the N112Q mutant produces a protein of 28 kDa (like hCtr1, see Figure 2.2), which is lowered to 23 kDa on treatment with PNGaseF. This establishes N112Q is glycosylated and that N15 is the site of attachment of N-linked glycosylation. N15 is thus exposed to the extracellular medium, supporting the model that places N112 in the cytoplasm (see Figure 2.4A).

Having established that the amino-terminal of hCtr1 is exposed to the extracellular medium, the presence of three putative transmembrane segments predicts an intracellular carboxy-terminus. In order to provide experimental support for this hypothesis we carried out labeling experiments on *sf9* cells expressing hCtr1 molecules bearing epitope tags at each terminus. It was first necessary to establish that these modifications did not produce inappropriately folded transporters. In Table 2.2, we show the results of Cu uptake assays carried out on *sf9* cells expressing N-terminal- and C-terminal-FLAG-tagged hCtr1 proteins. Clearly both constructs are able to mediate Cu uptake with K_m values that are unchanged by the substitutions. Thus it is reasonable to assume that these tagged molecules are appropriately folded. In Figure 2.5, we show that the amino-terminal FLAG-epitope is detected equally well in whole cells or membranes, i.e. whether or not the *sf9* cells are permeabilized prior to FLAG antibody addition. In contrast, the FLAG epitope is not detected at the carboxy-terminal of hCtr1 in whole cells, only if the cell membrane is disrupted by homogenization prior to antibody addition. These results confirm the extracellular location of the amino-terminus of hCtr1 that was strongly suggested by our glycosylation mutants described above, and establish that the carboxy-terminus of hCtr1 is in the cytosol as required if indeed there are an uneven number of transmembrane segments, as predicted by the hydropathy analysis.

2.4.3 *The role of the cysteine residues in hCtr1*

hCtr1 has only two cysteine residues. In many Cu-binding proteins coordination of the metal ion by sulfur centers are important. We explored the role of the cysteine residues in hCtr1 by replacing each of them with serines. In each case function was unaltered and Km values are obtained that are close to the values of unsubstituted hCtr1

(see Table 2.2), suggesting that metal coordination in hCtr1 does not involve these cysteine sulfur centers.

Western analysis of hCtr cysteine mutants revealed that these residues might be involved in multimer stabilization. Studies, with yCtr3 and hCtr1, have demonstrated the ability of these molecules to form multimers in the presence of a crosslinker (Pena, Puig et al. 2000; Lee, Pena et al. 2002). Similarly, wild type hCtr1, N15Q, N112Q and hCtr1 molecules containing a N-terminal FLAG epitope exhibited some retention of dimer formation even after migration through a SDS-PAGE gel in the presence of 6% SDS (see Figure 2.6). The cysteine-substitution mutants did not show this stability; C189S in particular showed only mobilities correlated with the monomeric species when probed with hCtr1 C-terminal antibody (Figure 2.6). The N-Terminal Flag construct showed predominantly the dimeric species (Figure 2.6) while the C-terminal Flag construct showed less dimer stabilization than other hCtr1 molecules (data not shown). When samples of hCtr1 were treated with β-mercaptoethanol (65°C) the presence of dimers was reduced significantly (Figure 6). β-mercaptoethanol treatment also reduced the presence of dimers for wild type as well as all other mutant forms of hCtr1 (data not shown).

2.4.4 *Conformational Changes in the M1M2 Loop*

Hydropathy analysis of hCtr1 suggests that the first transmembrane segment begins close to residues 59-63. This places the two Mets domains of the amino-terminus in the extracellular medium, appropriately placed for Cu coordination. Furthermore, this would place a single potential tryptic cleavage site (at K52) in the extracellular domain and several potential cleavage sites (K87, R90, R95, R102, and K121) in the first intracellular loop between transmembrane segments 1 and 2. In order to establish whether

or not this was correct we examined the effects of trypsin treatment on membranes isolated from *sf9* cells that expressed hCtr1. In Figure 2.7, we show the results of such experiments. Trypsin treatment of membranes produces a stable 10 kDa fragment that is visible after Western analysis using our C-terminal antibody. The size of this fragment is consistent with a cleavage at one of the more N-terminal arginines or lysines in the cytoplasmic loop between transmembrane segments 1 and 2 (see Discussion). In order to distinguish cleavage at K52 from intracellular cleavage sites, we compared the mobilities of the proteolytic 10 kDa fragment with that of an expressed truncation of hCtr1, beginning at V62. This polypeptide has an apparent mobility corresponding to 16 kDa (See Figure 2.7B). This is clearly significantly greater than the tryptic cleavage product of hCtr1. The presence of Cu during treatment with trypsin results in a protection against proteolytic digestion (see Figure 2.7). The digestion of a negative control, Na^+, K^+ ATPase, by trypsin was unaffected by the presence of Cu (data not shown). These data suggest that upon binding of Cu to the expressed protein, a conformational change in the M1M2 loop occurs that leads to protection of the tryptic cleavage site.

2.5 DISCUSSION

In the present work we have characterized the properties of hCtr1, the human Cu uptake protein, following its expression in *sf9* cells. We provide evidence in support of a three transmembrane segment topology, with an extracellular amino-terminus and an intracellular carboxy-terminus. The protein is expressed in a functional form in this system, and we have initiated an analysis of residues that are potentially important in to the molecular mechanism of Cu uptake.

In recent years, initially in yeast and more recently in mammalian systems (Valentine and Gralla 1997; Culotta 2001), it has become very clear that the cellular and organ levels of copper ions are highly regulated. Copper is an essential co-factor for a variety of cellular processes and at elevated levels is toxic to organisms. It is known that the proteins responsible for its removal, ATPases such as ATP7A and ATP7B, when disrupted result in severe human diseases, Menkes and Wilson diseases respectively (Bull, Thomas et al. 1993; Chelly, Tumer et al. 1993; Tanzi, Petrukhin et al. 1993; Vulpe, Levinson et al. 1993; Yamaguchi, Heiny et al. 1993; Petrukhin, Lutsenko et al. 1994). Relatively little is known about plasma membrane copper uptake mechanisms. Recently, the protein responsible for mediating copper uptake into human cells was identified and this protein was shown to complement copper uptake deficiency in yeast and to mediate copper uptake into mammalian cells (Zhou and Gitschier 1997; Kuo, Zhou et al. 2001; Lee, Prohaska et al. 2001). This protein is closely related to the Ctr family of proteins previously identified as mediating Cu uptake in S. cerevisiae (Dancis, Haile et al. 1994; Dancis, Yuan et al. 1994; Pena, Puig et al. 2000).

2.5.1 *Membrane topology of hCtr1*

The amino acid sequence of hCtr1 when examined by hydropathy analysis gives three clear transmembrane segments, which thus predicts either of the two topologies in Figure 2.4A. The present work was undertaken to establish which of these orientations was correct, in a system where the hCtr1 molecule was functional. Recently, we have optimized baculovirus infection of insect cells for the characterization of structure–function relations in the renal Na, K-ATPase (Hu, Eisses et al. 2000; Hu and Kaplan 2000; Gatto, McLoud et al. 2001) as well as ATP7B, the Wilson disease protein (Tsivkovskii, Eisses et al. 2002). In the present work, we show that this system is also appropriate for the functional expression of hCtr1 and that hCtr1 expression in insect cells mediates Cu uptake with a K_m of $\sim 3.5 \pm 1.3$ μM, which is close to the K_m values recently reported by Lee *et al* (Lee, Pena et al. 2002).

In order to distinguish between the two putative membrane orientations of hCtr1, we constructed substitution mutants at N15 and N112. Two different membrane orientations are predicted by hydropathy analysis of the hCTR1 amino acid sequence, one of the two consensus glycosylation sites would be extracellular (N15) and the other would be intracellular (N112) or vice versa Figure 2.4A. In both models, the first putative transmembrane segment separates N15 and N112. Only extracellular sites of plasma membrane proteins become glycosylated at N-X-S, N-X-T consensus sequences in the lumen of the endoplasmic reticulum. Our data show that the correct orientation for hCtr1 is the first of the two models shown in Figure 2.4A. Expression of the substitution mutants reveals that the N15Q mutant has a lower molecular weight than the other substituted mutant (N112Q) and that the apparent mass of N15Q (23 kDa) is unaffected by treatment with PNGase F, which removes glycosylation moieties. The N112Q

mutant, on the other hand, has the same molecular weight as the wild type protein and our cysteine-substituted mutants, and like these forms of the protein, its molecular mass is reduced by the treatment with PNGase F (Figure 2.4C). Thus our data clearly suggest the first orientation shown in Figure 4A depicts the membrane orientation of hCtr1.

Our transport data on the N15Q mutant also eliminate any essential involvement of protein glycosylation for the Cu transport process. It was earlier observed that yCtr1 was highly glycosylated and it was speculated that this glycosylation might play a role in transport (Dancis, Haile et al. 1994). This does not seem to be the case for hCtr1 in insect cells.

To further characterize this suggested topology, we used hCtr1 molecules that contained the FLAG epitope fused to either the amino- or carboxy-terminus of hCtr1. By probing cells that were expressing epitope-tagged hCtr1 with a Flag antibody before and after disruption, we could ascertain which end of the molecule was extracellular and which was intracellular. As seen in Figure 2.5, the FLAG antibody detects the amino-terminal epitope-tagged hCtr1 equally well whether labeled in whole cells or permeablized cells. This suggests that the amino terminus is located outside the cell in agreement with our glycosylation data. In addition, the carboxy-terminal epitope-tagged hCtr1 was labeled much more extensively in the permeablized cells as compared to the intact cells. This is the result we would expect if the FLAG epitope were located inside the cell. These data supply strong evidence that the first topological model in Figure 4A is correct.

hCtr1 is susceptible to trypsin treatment, containing at least one cleavage site within the cytoplasmic loop between transmembrane segments 1 and 2. This result lends

support to the topology that has been suggested by the other results in this paper. We have demonstrated that the amino terminus of hCtr1 is located extracellular and its carboxy terminus is intracellular. The loop between the first transmembrane and the second transmembrane segment is intracellular and has several putative tryptic cleavage sites. Trypsin treatment of hCtr1 in the absence of Cu produces a stable 10 kDa fragment. The trypsin cleavage site is most likely after the first transmembrane domain for the following two reasons. The 10 kDa fragment is a C-terminal fragment as our C-terminal antibody recognizes it. The predicted mass of a C-terminal fragment cleaved by trypsin after K52, the only extracellular cleavage site, is 15.4 kDa, 40% greater than the observed fragment. The predicted mass of a tryptic fragment cleaved after either K87 or R95 is 11.6 or 10.7 kDa respectively. The observed size of the C-terminal fragment clearly agrees most closely with cleavage at these intracellular sites. This reasoning is supported by our observation that a tagged truncation of hCtr1 beginning at V62 has an apparent mobility of 16 kDa (Figure 2.7B). Thus the cleavage site obtained with trypsin produces a smaller fragment than the predicted cleavage at K52 and is probably just after the peptide emerges from the membrane in the cytoplasmic loop between transmembrane segments 1 and 2. In the presence of 10 μM $CuCl_2$, the full-length hCtr1 molecule is protected from digestion suggesting that a conformational change in the cytoplasmic loop between transmembrane segments 1 and 2 has occurred following substrate binding. We are currently investigating the precise site(s) of cleavage, as this conformational change will be an important aspect of ligand-induced structural changes accompanying the transport process.

The membrane orientation we have determined has been assumed in previous work on yeast (Dancis, Haile et al. 1994; Dancis, Yuan et al. 1994; Pena, Puig et al. 2000) and mammalian Cu uptake proteins (Lee, Prohaska et al. 2000; Lee, Pena et al. 2002). Presumably this is because the proteins mediate Cu uptake and the presence of the MXMXXM motifs (Mets motifs) in the amino-terminus suggest that this may be the site of initial Cu binding by the protein. The present work puts this topology proposal on firm experimental ground.

2.5.2 *The role of cysteine residues in hCtr1*

In the other closely related yeast proteins, Ctr1 and Ctr3 (Pena, Puig et al. 2000), there are numerous cysteine residues that may play a role in Cu transport. hCtr1 has only two cysteine residues and our work shows that neither is essential for function. The K_m for each of the cysteine-substituted mutants is similar to wild type hCtr1 in our system (see Table 2.2). Neither of the two cysteines is conserved in other non-mammalian Ctr1 proteins. Therefore, our work and the lack of conservation of these residues in closely related Cu transport proteins, suggests strongly that Cu-protein interactions during transport must occur at other candidate Cu-coordinating residues, such as methionine (in Mets motifs) or histidine.

Additionally, our cysteine-substitution mutants do provide some evidence that the oligomerization of hCtr1 previously described in (Lee, Prohaska et al. 2000; Lee, Pena et al. 2002) may be mediated through interactions at its carboxy-terminal end. Because hCtr1 has only 190 amino acids and contains only three membrane segments, it has been suggested that the functional form of hCtr1 is probably a multimer. Pena *et al.* have shown that yCtr3 (Pena, Puig et al. 2000) and more recently hCtr1 does indeed form

multimers (Lee, Prohaska et al. 2000; Lee, Pena et al. 2002). In the presence of crosslinker, stable multimers of hCtr1 can be obtained. The structure and interactions of these multimers is unknown. We provide evidence that suggests the carboxy- terminus of hCtr1 may play a role in the formation and stabilization of functional hCtr1 complexes. hCtr1 molecules expressed in insect cells form stable oligomers even in the presence of SDS in Laemmli gels (see Figure 2.6). Treatment of NT-FLAG-tagged hCtr1 with β-mercaptoethanol dramatically reduces the amount of oligomerization seen in SDS gels (see Figure 2.6), implying that S-S bridge formation may be involved in stabilizing these multimers. In support of this, the C189S mutant shows only monomeric species in SDS gels (see Figure 2.6). However, since C189S is functional in Cu transport (see Table 2.2), the formation of such putative S-S bridges cannot be essential. In keeping with the idea that the C-terminal segment is involved in monomer-monomer interactions and stabilization of multimers, our CT-FLAG construct also shows predominantly monomeric species in SDS gels (data not shown), as if the epitope interferes with such interactions. Further experiments are underway to elucidate a functional role (if any) for multimerization. Currently, it appears that detergent stability of hCtr1 complexes may be facilitated by S-S bond formation, but such bonding is not essential for function. If functional hCtr1, by necessity, forms multimers, other interactions must occur to facilitate their formation in the native state.

The agreement in the apparent affinities of hCtr1 for Cu, following expression in mammalian cells and insect cells, coupled with the very low endogenous Cu uptake activity of *sf9* cells, indicates that the baculovirus-mediated expression in insect cells will be highly appropriate for the detailed characterization of hCtr1. In summary, we have

shown that hCtr1, the protein responsible for mediating copper uptake into human cells has an extracellular amino-terminus and a cytoplasmic carboxy-terminus (see Figure 2.8). Mutation of the two putative N-linked glycosylation sites or the two native cysteine residues has no significant effect on copper uptake in *sf9* cells. Mutagenesis reveals N15 as a glycosylation site for hCtr1. Additionally, our data suggest that the intracellular carboxy-terminus may be involved in forming stable multimers. This protein has few of the typical consensus metal-binding sequences associated with copper-dependent proteins and the heterologous expression of this protein in *sf9* cells offers the opportunity to begin to address important questions that relate to the mechanism of copper entry into mammalian cells, and the organization as well as structure-function relations in hCtr1.

2.6 ACKNOWLEDGEMENTS

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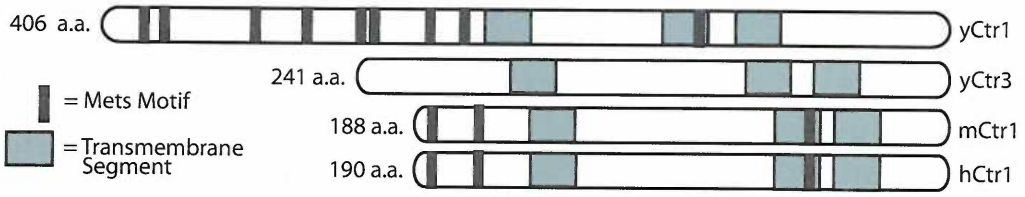
Figure 2.1 Representation of relative positions of putative transmembrane segments based on hydropathy analysis.

A) Yeast (Ctr1, Ctr3) and mammalian (mCtr1, hCtr1) copper uptake proteins. Methionine repeats are shown as black rectangles and transmembrane segments are shown as gray rectangles. The number of amino acids for each protein is also shown.

B) Kyte-Doolittle hydropathy plot of hCtr1 primary sequence is shown. Putative transmembrane segments are shown as a labeled line.

Figure 2.1

A


406 a.a.
yCtr1
241 a.a.
yCtr3
188 a.a.
mCtr1
190 a.a.
hCtr1
= Mets Motif
= Transmembrane Segment

B

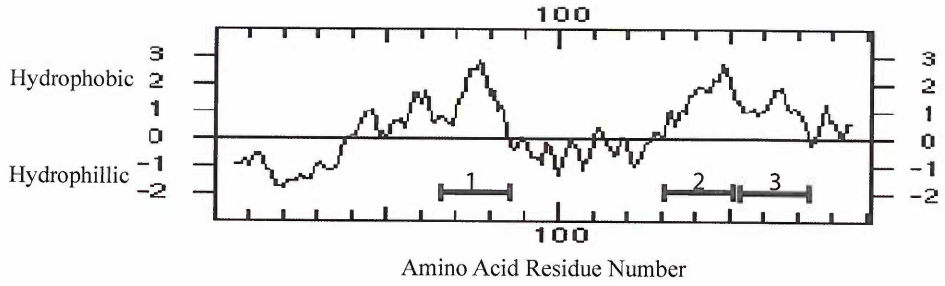
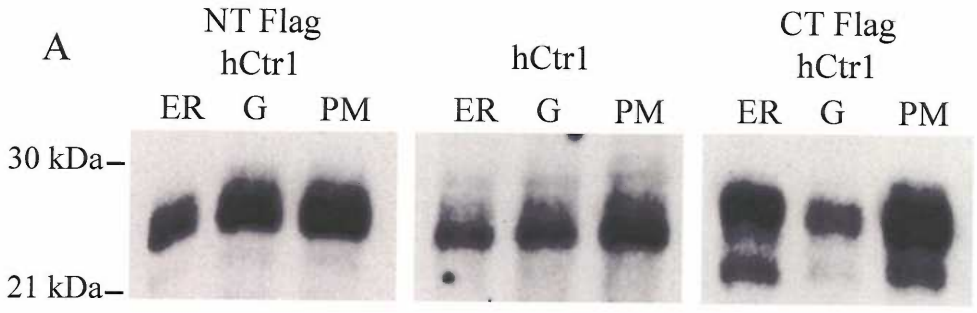

100
Hydrophobic
Hydrophillic
3
2
1
0
-1
-2
1
2
3
100
Amino Acid Residue Number

Figure 2.2 Sub-cellular fractionation of hCtr1 proteins.

A) *sf9* cells infected with hCtr1or AG epit ope-tagged fusion hCtr1 proteins were fractionated using a five-step sucrose gradient that generates endoplasmic reticulum, golgi and plasma membrane fractions. Western analysis was performed using 50 µg of membranes loaded on a 12% SDS-PAG gel. The blots we re incubated with C-terminal hCtr1 antibody (1:5000 dilution) and secondary detection was carried out using a goat anti-rabbit antibody (1:5000 dilution).

B) hFractionated membranes were prepared from Hep2Gcells and *sf9* cells expressing hCTR1 to investigate levels of endogenous (100 µg Hep2Gmembranes) and over-expressed hCTR1 (2 µg *sf9* membranes). The blot was probed with a rabbit anti C-terminal hCTR1 antibody (1:5000 dilution) followed by secondary detection using an HRP-conjugated anti rabbit antibody (1:5000). Analysis by densitometry suggests that expression in *sf9* cells produces 100 fold more protein than endogenous levels in Hep2Gcells.

Figure 2.2


A
NT Flag
hCtr1
hCtr1
CT Flag
hCtr1
ER G PM
ER G PM
ER G PM
30 kDa
21 kDa

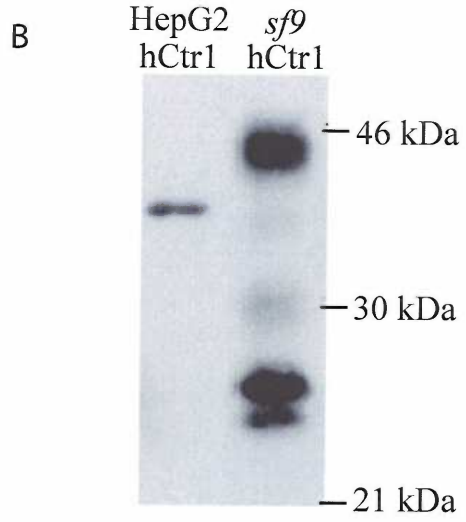

B
HepG2
hCtr1
sf9
hCtr1
46 kDa
30 kDa
21 kDa

Figure 2.3: Copper transport by cells expressing hCtr1.

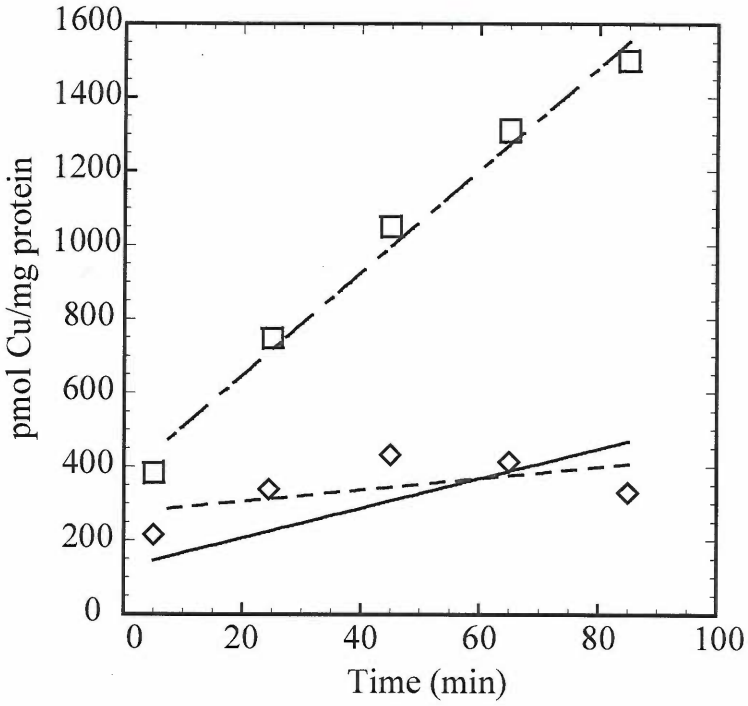
12 well plates were seeded with *sf9* cells (1.0 x10^6 cells /well). 72 hours post-infection, cell media were replaced with transport buffer (150mM NaCl, 2.5 mM $MgCl_2$, 25 mM Hepes, pH 7.4) and incubated at 27°C for 30 min. Cu transport was carried out using either ^{64}Cu or ^{67}Cu at specified $CuCl_2$ concentrations.

A) Time course experiments were performed at 10 μM $CuCl_2$. Shown here is an example experiment using uninfected cells (solid squares), *sf9* cells expressing the β subunit of Na^+, K^+ ATPase (open diamonds) or *sf9* cells expressing hCtr1 (open squares). hCtr1 mediates at least a 3-4 fold increase of copper uptake into *sf9* cells.

B) Saturable Cu uptake by *sf9* cells expressing hCtr1 is shown for a typical experiment. Different $CuCl_2$ concentrations were used to measure Cu uptake by uninfected cells (solid squares) or cells expressing hCtr1 (solid circles, K_m= 3.2 μM). Cells were lysed using 0.1N NaOH and aliquots were counted using a γ -counter (Packard Cobra II) and also used to determine protein concentrations. Expression levels of the hCTR1 mutants were monitored by Western analysis.

Figure 2.3

A


1600
1400
1200
1000
800
600
400
200
0
pmol Cu/mg protein
0
20
40
60
80
100
Time (min)

B

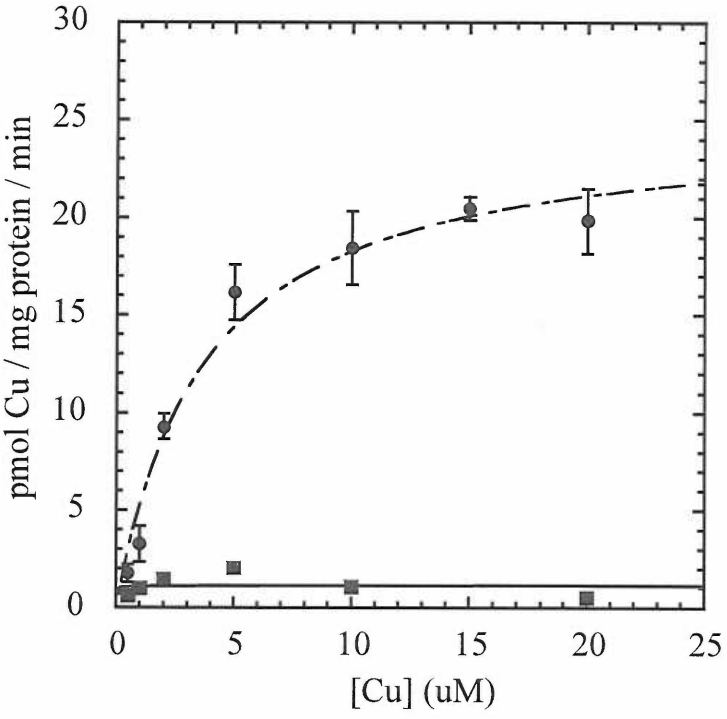

30
25
20
15
10
5
0
pmol Cu / mg protein / min
0
5
10
15
20
25
[Cu] (uM)

Figure 2.4 Alternative topological models for hCtr1 as determined by hydropathy analysis and enzymatic deglycosylation.

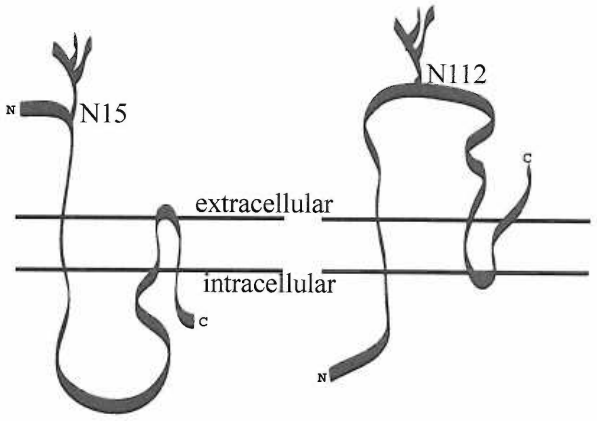
A). Two models can be predicted for hCtr; the first model has the amino terminus of hCtr1 located in the extracellular space and the carboxy-terminus located in the cytoplasm. The second possibility has the amino-terminus in the cytoplasm and the carboxy-terminus located in the extra-cellular space. In the first model, a potential N-linked glycosylation site (N15) is present in the extracellular space where it can be glycosylated. The second model shows a second N-linked glycosylation site (N112) to be in the extra-cellular space.

B). Copper transport data presented here for N15Q (solid circles, solid line) and N112Q (solid squares, dotted line) mutants demonstrate that mutation has no significant effect on Cu uptake by these proteins. The K_m values were 4.5 μM (N112Q) and 5.3 μM (N15Q).

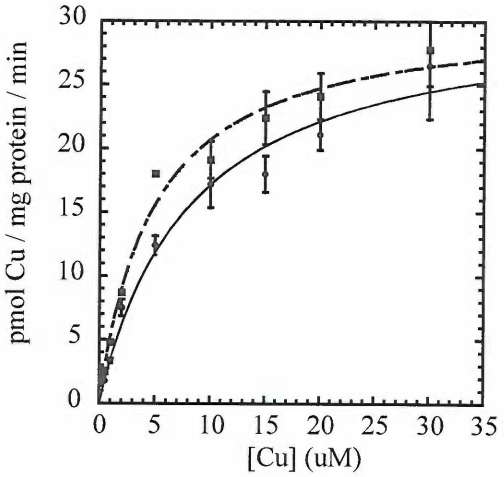
C). Mutant hCtr1 proteins N15Q and N112Q were expressed in sf9 cells for 72 hours, followed by membrane isolation. Membranes (30 μg) from each mutant sample were treated with PNGaseF (2.5 mU). Samples were run on a 12% Laemmli gel and analyzed by Western analysis. This provides evidence that hCtr1 is only glycosylated on N15 and that this amino acid is located outside of the cell.

Figure 2.4

A



B



C

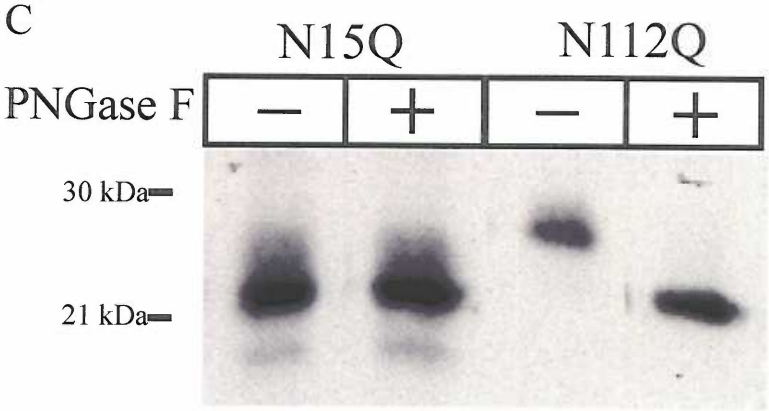


Figure 2.5 Anti –FLAG antibody labeling of epitope tagged hCtr1.

N-terminal and C-terminal epitope tagged hCtr1 expressed in sf9 cells were used to probe the amino- and carboxy-terminal topology of hCtr1. Intact sf9 cells were either surface labeled (IC) using a mouse monoclonal anti-FLAG antibody or used to prepare unfractionated membranes (M) for antibody labeling. Antibody labeling was carried out for 2 hours at 4°C for both samples. The IC samples were then used to prepare membranes. 500 μg of total membrane preps were solublized in 2% DDM buffer. The solublized membranes were then used in an immunoprecipitation as described in the experimental methods.

Figure 2.5

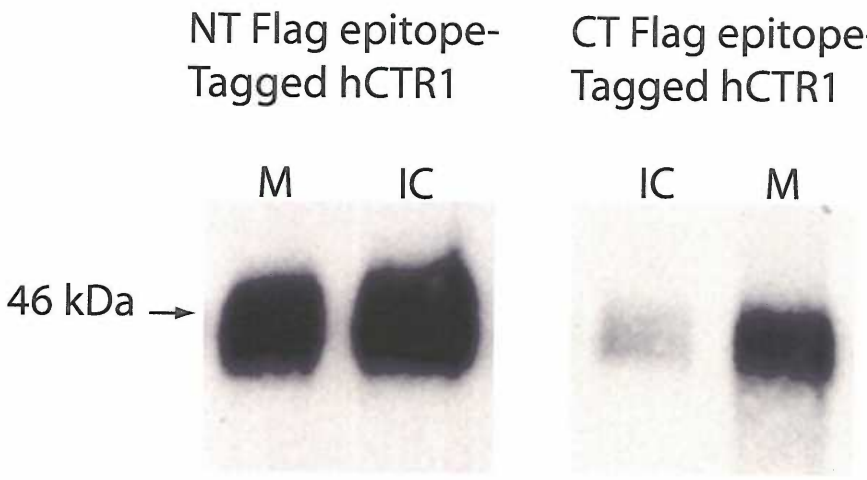

NT Flag epitope-
Tagged hCTR1
CT Flag epitope-
Tagged hCTR1
M
IC
IC
M
46 kDa

Figure 2.6 The role of cysteine residues in the formation of hCtr1 multimers.

N-terminal FLAG epitope-tagged hCtr1 and C189S hCtr1 (50 μg) were left untreated or treated with .2% β-mercaptoethanol, 65 °C, for 15 minutes in SDS sample buffer and then run on 12% Laemmli gels to investigate the role of cysteine residues in formation of multimers. Following Western analysis with anti hCtr1 antibody, the presence of multimers in lanes containing NT-FLAG hCtr1 is contrasted with the lack of multimers in C189S lanes. Addition of β-mercaptoethanol reduces the amount of multimerization in NT-Flag sample while leaving C189S sample unaffected.

Figure 2.6

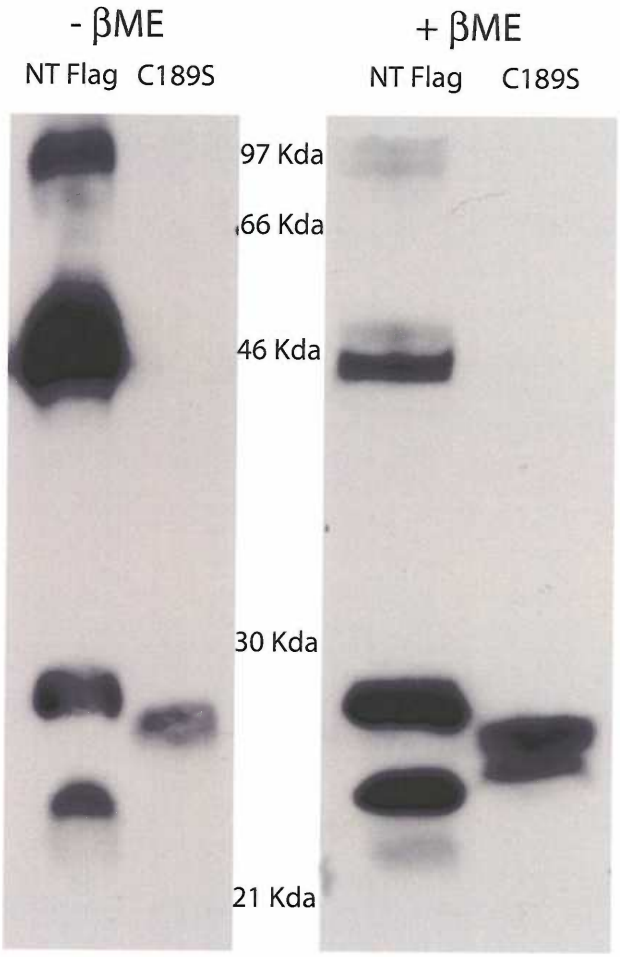

- βME
NT Flag C189S
+ βME
NT Flag C189S
97 Kda
66 Kda
46 Kda
30 Kda
21 Kda

Figure 2.7 Trypsin digestion of hCtr1.

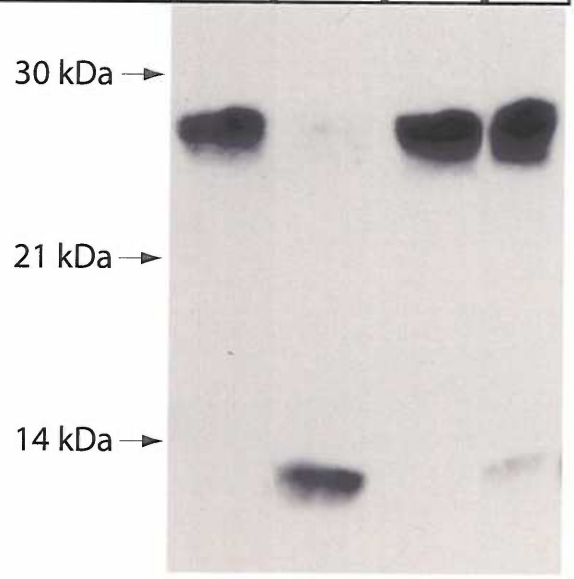
A) hCtr 1 membranes (C189S, 50 μg) were digested with trypsin for 60 minutes at room temperature in the presence and absence of 10 μM $CuCl_2$. The digestion was stopped by the addition of Soybean trypsin inhibitor and subsequent addition of sample buffer (6% SDS). Samples were run on 12% Laemmli and blotted to nitrocellulose membranes for Western analysis with anti C-terminus hCtr1 antibody.

B) To localize the potential tryptic cleavage site that produces the stable 10 kDa fragment seen in A, we expressed an epitope tagged N-terminal truncation hCtr1 molecule (V62-H190). It bears an amino terminal tag (MASYSHPQFEKGAETAVPNS) that can be used for detection. A 12% Laemmli gel was run with untreated C189S hCtr1 (lane 1), trypsin treated C189S hCtr1 (lane 2), and the N-terminal truncated hCtr1 (lane 3). The blot was analysed using a C-terminal hCtr1 antibody.

Figure 2.7

A

10 μM Cu	—	—	+	+
Trypsin	—	+	—	+
Time	0	60	0	60



B

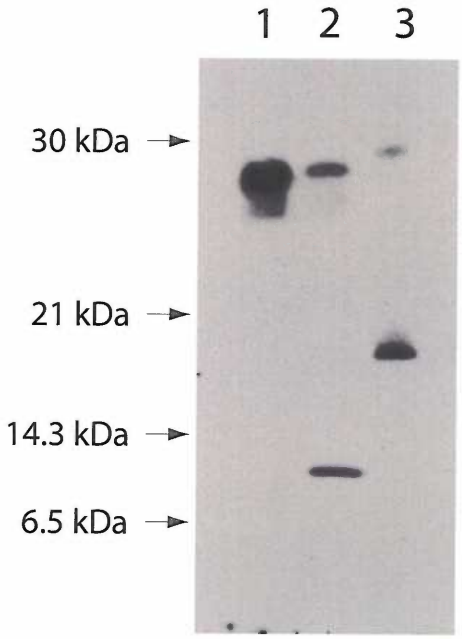


Figure 2.8 Model of hCtr1.

A topological model of hCtr1 is depicted in the plasma membrane with amino acid residues displayed with the amino-terminus extracellular and the carboxy-terminus intracellular. The N-linked glycosylation site as well as the methionine residues (red) is shown in the model. Mutated residues are depicted as yellow (N15, N112) or blue (C161, C189).

Figure 2.8

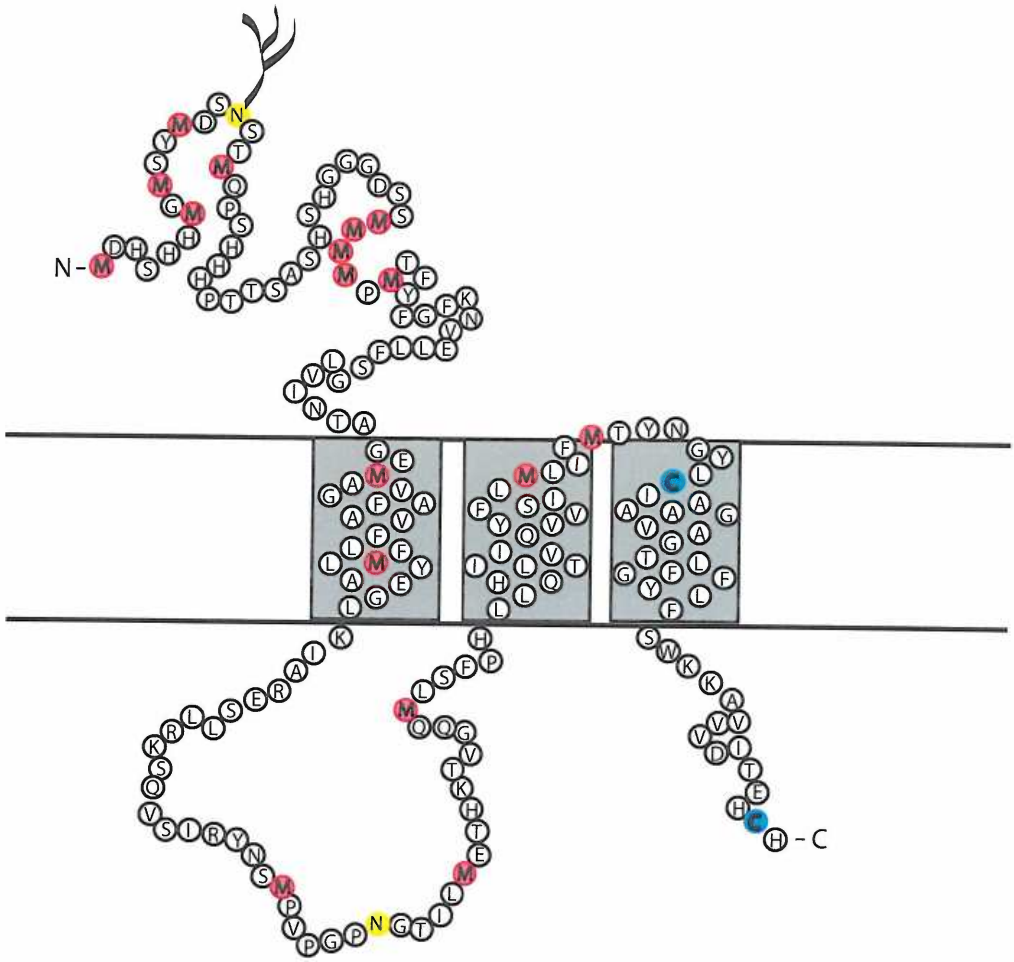


Table 2.1 Primer sequences for cloning and mutagenesis of hCtr1.

Primers were designed for generating and cloning different hCtr1 constructs into pFastBacDual vector. Sequences are given in a 5′ to 3′ orientation.

Table 2.1

Primer	Primer orientation	Nucleotide Sequence
N-terminal Flag hCTR1	Forward	5′-gaattcatggactacaaggacgacgatgacaagatggatcatcc-3′
	Reverse	5′-aagcttaacaacttccactgc-3′
C-terminal Flag hCTR1	Forward	5′-gactacaaggacgatgacggcaagtgacatcaaactc-3′
	Reverse	5′-cttgtcgtcatcgtccttgtagtcatggcaatgctc
hCTR1	Forward	5′-gaattcatggatcattcc-3′
	Reverse	5′-aagcttaacaacttccactgc-3′
N15Q	Forward	5′-gctatatggactccaaagtaccatgc-3′
	Reverse	5′-gcatggtactttgggagtccatatagc′
N112Q	Forward	5′-gcctgtccaggaccacaaggaaccatcc-3′
	Reverse	5′-ggatggttccttgtggtcctggacagge-3′
C161S	Forward	5′-cgggtacctctccattgcagtagcagt′
	Reverse	5′-gctgctactgcaatggagaggtaccg′
C189S	Forward	5′-cacagagcattcccattgacat-3′
	Reverse	5′-gatgtcaatgggaatgctctgtg′

Table 2.2 K_m Values for hCtr1 constructs.

Data from Cu uptake experiments at various Cu concentrations were analyzed using the Michaelis-Menten equation to determine the Cu concentration at which uptake was half maximal (K_m). K_m values were derived from multiple experiments (each experiment consisting of triplicate points) shown in the table. The standard error for the K_m values is shown. V_{max} values varied for different constructs (ranging from 20- 65 pmol Cu/mg protein/min) depending upon the expression level of the hCtr1 construct, the V_{max} value correlated with expression level as demonstrated by Western analysis.

Table 2.2

hCtr1 construct	Apparent K_m	(uM)	# of experiments
hCtr1	3.62	0.85	(4)
N15Q	5.90	1.53	(4)
N112Q	3.24	0.62	(5)
C161S	8.19	1.45	(4)
C189S	11.89	0.70	(4)
NT-FLAG	5.19	1.01	(5)
CT-FLAG	10.08	2.99	(3)

Chapter 3

The Mechanism of Copper uptake Mediated by hCtr1: A Mutational Analysis

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(Manuscript in preparation)

3.1 ABSTRACT

Copper entry into cells is extremely important for the efficient activity of copper containing enzymes. hCtr1 is a high affinity copper transporter thought to be the predominant uptake protein in mammals. The mechanism of hCtr1 mediated transport is not known. Candidate amino acids potentially important for transport have been elusive. Very little sequence identity exists between Ctr1 homologues. We report here the results of a mutational analysis of potentially important residues in the transmembrane domains. Methionine residues in the extracellular domain as well as the methionine motif in transmembrane 2 are critical for the transport of copper by hCtr1. The two cysteines when mutated individually have little or no effect on copper transport. However, a double cysteine mutant has a dramatic loss of transport activity. Two tyrosines found in the transmembrane domains have only modest affects on transport rate. Tyrosine 156 found in the short extracellular loop between transmembranes 2 and 3 appears to be important, as mutation to alanine dramatically decreases copper transport. Interestingly, the charged residues E68 and E84 have only modest effects on transport rate while H139R dramatically increases rate of copper uptake (10 fold). We present a working model for the role of these residues which have been shown to be important for transport activity mediated by hCtr1.

3.2 INTRODUCTION

Copper is an essential element that is required for many important cellular reactions that rely on the redox properties of this metal (Frieden 1986; Harris 2003; Sharp 2003). Like many of the essential trace metals, inappropriately high concentrations of Cu are toxic, both at the cellular and whole organism level. Thus copper homeostasis is tightly regulated so that sufficient copper is absorbed and excess copper is efficiently excreted.(Holm, Saraste et al. 1987; Howard, Sexton et al. 1998; Petris, Strausak et al. 2000; Jaksch, Paret et al. 2001; Medeiros and Jennings 2002; El Meskini, Culotta et al. 2003; Harris 2003; Richter and Ludwig 2003). Copper homeostasis at the cellular level involves the entry and exit of copper as well as the processes whereby copper is delivered to specific sub-cellular locations. Many of the regulatory mechanisms that govern the cellular localization of proteins involved in copper homeostasis are currently under investigation and progress has been made in understanding these pathways (Petris and Mercer 1999; Roelofsen, Wolters et al. 2000; Lutsenko and Petris 2003). However, investigations focused on the molecular mechanism of copper transporters, mediating both entry and exit of copper are still at an early stage.

Copper entry into cells may be mediated by either of two transporters, divalent metal transporter 1 (DMT1) and Copper transporter 1 (Ctr1). DMT1, a relatively less specific divalent metal ion transporter, is believed to play a role in copper (II) uptake, while Ctr1, a rather specific transporter for Cu (and perhaps Ag) is thought to mediate Cu (I) uptake. DMT1 has been shown to be involved in Cu uptake in the small intestine(Tandy, Williams et al. 2000), while Ctr1 has been shown to be essential for embryonic development (Kuo, Zhou et al. 2001; Lee, Prohaska et al. 2001). Presumably, the balance between these pathways for copper uptake will vary among cells and will depend upon their expression levels, ambient

pH (since DMT1 co-transports protons), their relative affinities for copper and the oxidation state in which the metal ions are delivered to the transporters. The current work focuses upon the mechanism by which Ctr1 mediates copper entry.

In yeast, two copper uptake proteins are functional, yCtr1 and yCtr3. In humans, a single Cu (I) transporter is responsible for copper uptake at the plasma membrane (Zhou and Gitschier 1997{Lee, 2002 #233). This protein, hCtr1, consists of 190 amino acids containing three putative trans-membrane segments, and it has been shown to have an extra-cellular amino terminus and its carboxyl terminus localized within the cell (Eisses and Kaplan 2002). hCtr1 contains a single N-linked glycosylation site and it has been speculated that it may also contain O-linked glycosylation. The protein has many putative copper binding ligands including cysteines, histidines, methionines and tyrosines in its primary structure. There have been only relatively few structure-function analyses of hCtr1. The dominant features of the protein which may provide clues to its mode of action are, the two methionine-rich sequences in its extra-cellular amino-terminus, the presence of putative copper-coordinating residues within the trans-membrane segments, and its documented propensity for relatively stable oligomerization (Lee, Pena et al. 2002; Puig, Lee et al. 2002; Aller, Eng et al. 2004{Eisses, 2002 #206).

The two cysteines (C161 and C189), found in the carboxyl-terminal portion of the protein, can be removed without loss of function (Eisses and Kaplan 2002). The methionines in the extra-cellular domain (M40-M45) and in the second trans-membrane domain (M150, M154) have been shown to affect the ability of the protein to transport Cu into the cell (Puig, Lee et al. 2002). The roles of the other potential Cu binding ligands in hCtr1 have not yet been investigated. Residues in the third trans-membrane domain have been shown to have

structural importance. The glycine residues G167 and G171, are suggested to be involved in important helix packing interactions and oligomerization of hCtr1 multimers (Aller, Eng et al. 2004). Additionally, Aller et al used tryptophan scanning of the third transmembrane segment of yCtr3 to provide evidence that other residues (I196, I197, S198, C199 and R207) may have a role in protein folding and structural integrity. Because of the sequence homology between Ctr3 and hCtr1, these residues may also be important for hCtr1as well.

We have utilized over-expression of hCtr1 in insect cells to begin to carry out a structure-function analysis and determine the roles that specific amino acids might play in the transport mechanism. We have utilized measurements of radio-isotopic copper uptake by cells expressing mutant forms of hCtr1 to examine the potential roles of amino acids within the trans-membrane domains as well as the roles of both the amino- and the carboxyl-terminus. We combine our results with those of previous studies to propose a model of how hCtr1 mediates copper entry into cells.

3.3 Methods

3.3.1 *Cell maintenance*

Sf9 cells were cultured in Ex-Cell 420 media (JRH Biosciences, Lenexa, KS). Cells were maintained attached in t-75 flasks and were split when 90% confluent. Mutant hCtr1 constructs were maintained in Ex-Cell 420 media that was supplemented with 0.015 mg/ml Blasticidin S (Invitrogen, Carlsbad Ca.) to ensure retention of expression vector.

3.3.2 *Generation of mutant hCtr1 constructs*

hCtr1 constructs were PCR-amplified using outside primers (5' forward primer: 5'-gaattcatggatcattccc-3', and 3' reverse primer: 5'- ccgcggaacaacttcccactgc-3') that contain EcoR I and Sac II restriction sites engineered at the 5' and 3' ends respectively and internal primers specific for the mutation generated (Table 3.1) using *pfu* polymerase (Stratagene, La Jolla, Ca.) and ligated into pIB/H5 TOPO using the TOPO technology developed by Invitrogen, Carlsbad, Ca. The two C-terminal truncations were PCR-amplified using a construct containing asparagine-15 mutated to glutamine (Eisses and Kaplan 2002). Mutations were confirmed by sequencing the entire gene. Each pIB vector containing different mutations was transfected into sf9 cells using Cellfectin reagent following the manufacturer's protocol. Cells were selected for integrated vector using 80μg/ml Basticidin HCl for two weeks. Each stable cell line was checked for hCtr1 protein expression using either a C-terminal hCtr1 antibody (20 μg membrane protein; 1:25,000) (Eisses and Kaplan 2002; Eisses, Chi et al. 2005) or in the case of the two carboxyl-terminal deletions, an antibody raised against the N-terminal peptide (SHHHPTTSASHSHG) was employed. A feature of the proteins expressed in stable cell lines is the relative lack of higher order multimers compared with virally infected cells(Eisses and Kaplan 2002). Only under conditions of high protein concentrations loaded on protein gels do dimeric or trimeric hCtr1 complexes seem to be detected.

3.3.3 *Cu-64 uptake experiments*

Experiments to determine the ability of mutant hCtr1 proteins to transport copper were carried out as previously described (Eisses and Kaplan 2002) with slight modifications. Briefly, Copper uptake was performed in 12 well plates seeded with $1x10^6$ cells per well and allowed to incubate at 27°C with cell media. After the cells had attached, they were washed with uptake buffer (150mM NaCl, 2.5 mM $MgCl_2$, 25 mM Hepes, pH 7.4) and incubated at 27°C for 30 min. To initiate transport experiments, cells were incubated with Cu-64 in uptake buffer for 5min or 45 min at six different copper concentrations (0.5, 1.0, 2.0, 5.0, 10.0, 20.0 μM $CuCl_2$). Cu transport was carried out using ^{64}Cu (MIR Radiological Sciences). Cells were washed 3 times with ice-cold incubation buffer containing 10 mM EDTA. Cells were lysed using 0.1N NaOH and aliquots were counted using a γ -counter (Packard Cobra II) and also used to determine protein concentrations. Expression levels of the hCTR1 mutants were monitored by Western analysis. Radioactive Cu uptake was calculated as previously reported (Eisses and Kaplan 2002), essentially the amount of radioactivity remaining in contact with the cells, either bound to proteins at the surface or taken into the cell is used to calculate transport. Radioactivity incorporated into the cell is multiplied by the extracellular copper concentration and divided by the total radioactivity added to start transport. This amount is normalized by the protein concentrations for each well. This helps to normalize the uptake measurements in relation to fluctuations in cell number at the start of transport and loss of cells during washes to remove excess copper. These calculations allow us to calculate the number of pMoles Cu per mg of protein *min.

3.3.4 *Western blot analysis*

Cells were collected and lysed using a Dounce homogenizer to produce "total" or fractionated membranes containing over-expressed hCTR1, following previously published protocols for sucrose gradient centrifugation(Eisses and Kaplan 2002), and analyzed by Western blot analysis. Western analysis was performed using 20 μg of membrane protein loaded on a 12% SDS-PAGE gel. The gel was electro-blotted to a nitrocellulose membrane and blocked using PBS, pH 7.4 containing 5% milk. After blocking, the membranes were incubated with C-terminal antibody (1:25000, PBS pH 7.4, 0.5% Tween 20, 0.5% milk) and secondary detection was carried out using a goat anti-rabbit antibody (1:10,000, PBS pH 7.4, 0.5% Tween 20, 0.5% milk).

3.4 RESULTS

We have engineered mutations of many of the conserved residues in the trans-membrane domains of hCtr1 (Figure 3.1) as well as made truncations of both the amino- and carboxyl termini and have stably expressed these mutants of hCtr1 in insect cells. We show that a number of these residues in the trans-membrane domains have functional consequences for the transport of copper across the membrane. Specifically histidine 139 and tyrosine 156 have significant roles in the functional uptake of copper. The carboxyl terminus is also important for transport of copper into the cell.

In Figure 3.2, we show the expression levels of two hCtr1 constructs in stable cell lines; both Wt and C189S hCtr1 proteins traffic to the plasma membrane. Most of the expressed protein is found in the plasma membrane fractions where it is expected to facilitate the uptake of copper into the cells. In plasma membrane fractions, three protein bands are observed following antibody staining, a 28 kDa band corresponding to glycosylated hCtr1 monomer, a 24 kDa band corresponding to non glycosylated hCtr1 and a carboxyl-terminal degradation product of 17 kDa. No protein band is revealed in any of the fractions isolated from sf9 cells lacking hCtr1 expression vectors.

3.4.1 *Expression and function of truncation mutants*

We cloned three amino-terminal deletions of hCtr1 to investigate the potential role of the extra-cellular domain in facilitating copper transport. We investigated the role of these domains by expressing protein constructs that were truncated just before the second methionine repeat (MG34) or after this methionine repeat (MN53 and M69) (Figure 3.3). Each of the three amino terminal truncations expressed well and was localized to the plasma membrane in *sf*9 cells. Copper uptake facilitated by MG34 was similar to Wt levels (see

Figure 3.3 and Table 2; Km ≈ 14.0 ± 2.2, Vmax = 84.1 ± 7.1). The two truncations, MN53 and M69, in which this 2nd extra-cellular methionine repeat was removed, were significantly impaired in their ability to facilitate copper uptake (Km ≈ 3.5 ± 0.4, Vmax = 29.8 ± 1.3; Km ≈ 2.4 ± 0.7, Vmax = 18.0 ± 1.5, respectively). The Vmax for M69 is very similar to that for sf9 cells that have no over-expression of hCtr1. Interestingly, in the case of the MN53 truncation mutant, a reduction in copper transport is observed, around 25% of the capacity of the Wt protein but not complete loss of all transport as reported by others(Puig, Lee et al. 2002, see Discussion). The Km values for copper transport do not seem to be affected significantly in any of the amino terminal truncations (see Table 3.2).

We also investigated the potential importance of the carboxyl-terminal intra- cellular tail of hCtr1 to facilitate copper transport. We have shown previously, in baculovirus-infected sf9 cells, that the carboxyl-terminus can be epitope-tagged without significant disruption of hCtr1 function (Eisses and Kaplan 2002). However, it is not known if residues in the carboxyl-terminus play any role in copper uptake. Two carboxyl-terminal truncations were generated; CTK178 and CTD184 (see Figure 3.3). CTK178 is a construct in which hCtr1 is terminated at K178 and CTD184 is a construct in which hCtr1 is terminated at D184. Both of these constructs are expressed in sf9 cells at comparable levels and are delivered to the plasma membrane (Figure 3.3) and are able to mediate copper uptake although at reduced levels (Figure 3.3 and Table 3.2). The transport capacity of the expressed CTD184 is about 45% of the level seen in cells expressing Wt hCtr1. Removal of additional amino acids reduces the capacity for copper uptake further (30% of Wt hCtr1). Both carboxyl-terminal protein truncations thus have lower Vmax values, but the apparent Km's for copper uptake mediated by these two constructs remains similar to Wt hCtr1.

3.4.2 *Putative Copper coordination mutants in the trans-membrane domains*

Within the three trans-membrane domains, there are four methionine residues, M69, M81, M150 and M154. Two of these methionines are conserved in every high affinity copper transport protein of the Ctr family (see Figure 3.1). We expressed a double mutant, M150I/M154I, to investigate the effects of this set of mutations in our insect cell expression system. We see limited but reproducible uptake of copper across the plasma membrane of sf9 cells expressing the double mutant, M150I/M154I (see Table 3.2). Copper uptake by the double methionine mutant is about 30% of the uptake seen by Wt hCtr1 in the same experimental system. Protein levels of the M150I/M154I mutant are similar to the levels of Wt hCtr1 at the plasma membrane (data not shown).

We also investigated the copper transport facilitated by two other methionine mutants, M69I and M81I. These are the only other methionine residues in the putative trans-membrane segments, both of these methionines are in the first trans-membrane segment. These two mutants are capable of transporting copper into the cell at higher levels than the double mutant M150I/M154I. Copper transport by M69I and M81I is slightly reduced as compared to Wt hCtr1, 79% and 54% respectively. The protein expression of M69I is lower than either M81I or M150I/M154I mutants at the plasma membrane (Figure 3.4). The majority of the M69I protein still traffics to the plasma membrane as with other mutants, suggesting that the relative decrease in copper uptake by this mutant is likely due to the lower level of expression and not misfolding or retention in the endoplasmic reticulum.

We showed earlier that C161 and C189 were not likely essential for copper transport. When each of these residues was mutated individually the proteins were capable of transporting copper with a similar apparent affinity as Wt hCtr1 and with comparable Vmax

values (Eisses and Kaplan 2002). In the present work we examined the double mutant. Surprisingly, when both cysteine residues are mutated to serines, we see a dramatic effect on the ability of this construct to transport copper across the membrane. The apparent Km for this mutant is similar to the apparent Km for Wt hCtr1 (8 μM, compared with 13.5 μM, see Table 2). The Vmax for C161S/C189S, however, is only 34% of the Vmax seen with the Wt hCtr1 construct, at comparable levels of protein expression.

3.4.3 *Tyrosine mutants in the trans-membrane domains*

There are two strictly conserved tyrosine residues (Y83 and Y147, in human) in the trans-membrane domains of yeast and mammalian forms of Ctr, (see Figure 3.1). A third tyrosine residue, Y156, is located in the putative extra-cellular loop between trans-membrane segments 2 and 3. We investigated whether these three tyrosines are important for copper transport mediated by hCtr1. Y83 and Y147 were mutated to either alanine or a phenylalanine and stably expressed in *sf*9 cells. Protein expression and copper uptake were characterized for each mutant (Figure 3.5). In both cases, the Y->A mutant transported with a higher Vmax than the Y->F mutant (Table 3.2), suggesting perhaps that the size of the amino acid side-chain impacted transport. The Km values for each mutant were similar to Wt hCtr1. In each case the mutants Vmax values were ~40% of WT for the phenylalanine mutants and ~60% of Wt for the alanine mutants of these two tyrosine residues suggesting these residues may be important for transport of copper (Table 3.2).

The Y156A mutation had a more dramatic effect on copper uptake (Table 3.2). The mutant protein was expressed at comparable levels as Wt but the Vmax is only 26% of the Vmax of the Wt construct. The apparent Km of Y156A is only slightly higher that Wt hCtr1 in our expression system. The low Vmax value, which is similar to the double methionine

M150I/M154I mutant, suggests that Y156 may be important for the translocation of copper through the membrane (see Discussion).

3.4.4 *Charged residues in the trans-membrane domains*

There are three charged residues in the trans-membrane segments of hCtr1, E68, E84 and H139. The occurrence of charged residues in the trans-membrane segments of cation transport proteins is often associated either with important structural or functional roles, since such residues are normally more stable in highly polar environments. We mutated the two glutamic acid residues to either a leucine, which has a nonpolar side chain, or to a glutamine, having an uncharged polar side chain. The mutant proteins were expressed equally well and were delivered to the plasma membrane (Figure 3.6B). Interestingly, the mutant proteins transported copper at slightly higher rates than the wt hCtr1. E68L and E68Q transported copper at 150% and 127% of the Wt hCtr1 rate, respectively. The Km values for these two mutants were only slightly higher compared with the Km value for Wt hCtr1 (Table 3.2).

The Km value for the E68Q mutant is only 2 times higher than the Km value for Wt hCtr1 in this expression system. Similarly, the mutants E84L and E84Q also transported copper at higher rates than Wt hCtr1 (112% and 150% respectively). The apparent Km values show slightly lower affinity for copper than Wt hCtr1

The most dramatic effect seen with any of our mutants occurs when the histidine at position 139 is mutated to an arginine. When H139 is mutated to an arginine the apparent Km for this mutant is close to 50 μM, an approximately ten-fold decrease in affinity compared with wt hCtr1 protein. Interestingly, the Vmax for this mutant is also dramatically affected with a Vmax of 271.5 ±20.2 pMoles Cu/mg protein*min, that is about 4.5-fold higher than the Wt transporter. Protein expression and trafficking of this mutant is similar to

Wt hCtr1 and other mutant proteins (Figure 3.6b). When H139 is mutated to an alanine a different effect on transport is seen. The apparent Km value stays similar or slightly higher than Wt hCtr1. The Vmax for this mutant is reduced to about 50% of the value we see for Wt hCtr1 (Table 3.2). The presence of a positive charge at this position in the protein appears to have a significant effect on the apparent affinity for copper uptake by this mutant as well having a dramatic effect on the rate at which copper is transported into the cell.

3.5 DISCUSSION

In the present work we have carried out a structure-function analysis of hCtr1 in order to identify important amino acid residues in this transport protein. Residues that affect transport can provide information that allows a working model of how hCtr1 mediates copper entry into cells to be formulated. We have generated stable insect cell lines that express Wt hCtr1 and a number of point mutations and truncations. The majority of single residue replacements that we have examined produce only subtle changes in the transport properties of hCtr1. More dramatic changes are observed with the deletion of the amino-terminal methionine-rich segment and when a positively charged residue is placed within the membrane in the second transmembrane domain. It is striking and important that there are no essential residues identified within the membrane whose alteration leads to a complete lack of functional activity. This leads us to propose a model in which there is a pore-like functional structure for this transporter (see below).

Stably expressing insect cells provide several advantages over the baculovirus expression system previously employed (Eisses and Kaplan 2002). One of the major advantages is that consistent expression of the protein of interest is achieved once the cell line has been established. Baculovirus-mediated expression uses viral infection of the insect cells and requires the use of a viral isolate to initiate expression of the heterologous protein. It is very difficult to ensure, even using the same viral stock that the infection of different populations of cells will result in a uniform and reproducible pattern of protein expression. Furthermore, when the expressed protein of interest is a transport protein, it is more critical that membrane integrity of the infected cells be maintained. This is difficult when the infecting agent is a lytic virus. A stable cell line alleviates these difficulties, and provides a

permanent and highly reproducible source of the transport protein in uncompromised cells membranes.

A second difference between these two insect cell expression systems is the level of protein expression for each mutant. In stable cell lines, hCtr1 mutant proteins were expressed at slightly lower levels than the corresponding virally induced mutant proteins (data not shown). This is to be expected as transcription is driven by different promoters in the two expression systems. The stable cell lines use a baculoviral immediate early promoter that gives consistent but lower protein expression as compared to the late promoters (Jarvis, Weinkauf et al. 1996). This promoter is constitutively active and does not require any viral components to drive expression. In contrast, the promoters used for viral infection and subsequent protein expression utilize very late promoters and require virally encoded proteins to drive expression. In addition to the expression levels of the promoters, a second issue that may promote the differences we see between these two insect cell expression systems is the fact that the integrity of cellular membranes becomes compromised. Membranes contain large amounts of viral protein as well as the overexpressed protein of interest. At the time cells are collected to harvest expressed protein the cells are very sick and many of the cellular processes can be severely compromised. Interestingly, while protein levels (estimated from Western blots) from the two systems varied (virally-mediated expression produced higher levels of protein), the stable hCtr1 cell lines expressing wt hCtr1 exhibited higher levels of copper uptake than the sf9 cells that expressed hCtr1 following viral infection. The Km values for the copper uptake mediated by Wt protein were similar, whether expressed in the viral system or in cells stably expressing hCtr1. This suggests that while more protein was expressed with the viral system, a larger fraction of the protein was

not active at the plasma membrane. Protein levels in the plasma membrane fractions of stable cell lines were lower than protein in the plasma membrane fraction of the viral expression system. This may suggest that inactive protein is capable of localizing to the plasma membrane in the viral expression system.

3.5.1 *Expression and function of truncation mutants*

Two of the truncations we have generated have been described previously (Puig, Lee et al. 2002). Puig *et al* showed that the second methionine motif (M40-M45) was important for copper uptake, specifically the residues M43 and M45 were reported to be to be essential. Puig et al measured copper uptake at a single time point and reported that after 5 min the uptake of 2 μM copper was greatly reduced compared with wt hCtr1, reduced to a level similar to that of cells transfected with an empty vector. They suggested that this motif plays an important role in copper uptake, specifically involved in the "sensing" of extra-cellular copper. It has been suggested that the extra-cellular amino-terminal domain is require under copper limiting conditions and this has been shown by growing ctr1Δctr3Δ yeast cells in the presence of bathocuproine disulfonic acid (BCS), a copper chelator.

We also see the second methionine repeats importance for radio-isotopic copper uptake, the rate at which copper enters the cell is severely reduced. The truncation mutant MN53 rate of isotopic Cu uptake is only 25% of Wt. While this signifies that the amino terminal portion of hCtr1 is important, copper is still transported into cells. The affinity of this truncation for copper does not appear to be affected or may have a slightly higher affinity for copper. A strength of our results is the reporting of the affinities of various mutants for copper as well as reporting the relative rates of copper transport mediated by these mutant hCtr1 proteins.

Importantly, the loss of the entire extra-cellular domain (residues M1 to X69) results in a complete loss of copper uptake. This suggests that the residues that reside between the second methionine repeat (M40-M45) and the first trans-membrane domain (beginning approximately at G67) are crucial for copper transport. Several possible reasons for the importance of this region can be presented. It has been claimed, via yeast two hybrid assays, that the amino terminus is able to interact with itself and therefore may be involved in functional oligomerization of monomers (Klomp, Juijn et al. 2003). The amino acids in this region of hCtr1 (residues F47-A66) are highly hydrophobic in many of the Ctr1 proteins that are available for protein alignment (11 hydrophibic residues out of 20in hCtr1, Figure 3.1A). It is not known whether these residues of hCtr1 are associated with the membrane or are used to provide structural constraints for the extracellular domain of hCtr1. It seems most likely that the associations among the amino-terminal segments at the outside surface of the plasma membrane brings together important copper-coordinating residues that serve to steer or accumulate copper in the vicinity of a pore formed between the monomeric units (see below).

The carboxyl terminal truncations of hCtr1 were unable to transport copper at wt hCtr1 levels. The Km values are similar to wt hCtr1 but the rates of transport for the two mutants were less than half the rate of full length hCtr1. It has been suggested that the carboxyl-terminus is important for formation of hCtr1 multimers (Eisses and Kaplan 2002; Aller, Eng et al. 2004). Since most transporters have between 6-12 transmembrane segments and given that hCtr1 can form higher order oligomers (Pena, Puig et al. 2000; Eisses and Kaplan 2002; Lee, Pena et al. 2002; Aller, Eng et al. 2004), the functional hCtr1 complex is thought to be a multimer. If the functional hCtr1 complex requires stable oliomeric complexes for function, then anything that destabilizes this complex may have an effect on

the rate of Cu transport mediated by hCtr1. Interestingly, many of the mutations that seem to disrupt stable complex formation are found in the third transmembrane segment or in the carboxyl terminal portion of the protein (Eisses and Kaplan 2002; Aller, Eng et al. 2004). This suggests that the carboxyl terminal portion of the protein may play a role structurally, stabilizing a functional multimer complex. Additionally, we can not rule out that the carboxyl terminus of hCtr1 plays a role in the transfer of copper to intra-cellular copper homeostatic components, and that this transfer is a requirement for efficient transport. Very little is known concerning the immediate fate of copper as it enters the cell. It has been speculated that copper chaperones accept copper from Ctr1 proteins. There is report that claims the carboxyl terminus of yCtr1 can interact with Atox 1 and copper has been shown to transfer between these two proteins in vitro (Xiao and Wedd 2002; Xiao, Loughlin et al. 2004). If copper transfer to intracellular components is required for hCtr1 to function efficiently then mutations in regions responsible for copper transfer could affect copper uptake mediated by the Ctr1 family of transporters.

3.5.2 *Putative copper coordination sites in the trans-membrane domains*

In our experiments, M150 and M154 are important for the transport of copper, however they are not essential. We see radio-isotopic uptake of copper at 28% of wt hCtr1 when these residues are replaced with isoleucines. While this underscores the importance of these two amino acids, the M150I/M154I double mutant hCtr1 is capable of transporting copper into the cell.

Puig et al have suggested that the two methionine residues (M150, M154) in the second trans-membrane domain are essential for copper transport (Puig, Lee et al. 2002). Puig et al showed by complementation as well as copper uptake into yeast or mammalian

cells that mutations at these two sites have dramatic effects on copper uptake. Interestingly, the effects on complementation and uptake are not always to the same degree or have the same effect with Ctr1 molecules from different species. Puig et al measure uptake at a single time point for a single copper concentration (2 μM). Our experimental protocol for our uptake experiments differ from Puig et al in the timing of the measurements for uptake. We have measured copper uptake for 40 minutes at multiple copper concentrations. If a mutant Ctr1 protein transports copper at a very slow rate compared to wt hCtr1 then a single early measurement may not reveal a small but significant transport capacity. In addition it is important to be able to distinguish effects on the apparent affinity from effects on the transport capacity. Such experiments require that a range of substrate concentrations be employed.

Mutations of the two methionine residues (M69I and M81I) show small reductions in the transport of copper into the cell (M81I more than M69I); however, they are not reduced to the same extent as the double M150I/M154I mutant protein.

3.5.3 *Putative role of the two cysteines in hCtr1*

Interestingly, the double mutant C161S/C189S is significantly reduced in its ability to transport copper. This is surprising because neither of the single mutations caused a significant reduction in the ability of hCtr1 to transport copper into the cell. A possible explanation of this result is that mutations in the carboxyl terminus ie. C189 affect multimerization of hCtr1 mildly, however secondary mutations in the third trans-membrane domain exacerbate this effect. Experimental support for this suggestion can be found in the work of Aller et al (Aller, Eng et al. 2004). They show by tryptophan scanning mutagenesis of the third trans-membrane domain that appropriate helix packing of the protein is adversely

affected by bulky groups in the region of C161. Therefore if both regions are important for helix packing and for structural integrity allowing efficient multimerization of Ctr1 proteins, multiple mutations in these regions may significantly disrupt the ability to provide a transport pathway for copper (see discussion on carboxyl terminal truncations above).

3.5.4 *Tyrosine mutants in the transmembrane domains*

It is known that tyrosine residues are important structurally as hydrogen bond partners in proteins and can also participate in metal ion coordination (Arnesano, Banci et al. 2003{Whittaker, 2005 #583; Whittaker 2005).We observed only small reductions in rate of copper transport when Y83 or Y147 were replaced by alanine residues. Side chains that were bulky (phenylalanine) but did not contain an –OH group had a larger reduction in the proteins ability to transport copper than relatively small hydrophobic groups like alanine. This was seen when Y83 and Y147 were mutated to phenylalanine as compared to alanine. This may suggest that hydrogen bonding by these tyrosines can help stabilize the monomeric protein or the formation of a functional complex by multimeric hCtr1 protein.

The mutation of Y156 to alanine had a dramatic effect on the transport of copper mediated by hCtr1. Y156A only transported at a rate of about 25% of wt hCtr1. This residue is probably in the short loop between trans-membrane domain 2 and 3, although a detailed knowledge of the precise topology is limited. This residue is very close to methionine 154 at the extra-cellular boundary of the putative transport pore. Tyrosine 156 may play a structural role helping to stabilize methionine 154 for proper coordination of copper ions as they pass through the translocation pore.

3.5.5 *Charged residues in the transmembrane segments*

There are only three charged residues in the trans-membrane domains of hCtr1. These residues are located in the first trans-membrane domain (E68, E84) and the second trans-membrane domain (H139). We see modest increases in the transport rates for the two glutamic acid residues when they are replaced with uncharged residues. This is surprising because rarely are charged residues found in transmembrane segments that have little effect on protein function when mutated. The modest increases in transport rates may signify a role in copper movement through the membrane. Interestingly, the only mutations that seem to increase the rate of transport by hCtr1 are mutations involving the three charged transmembrane residues. Not only is the rate of transport increased, the apparent affinity of these mutaions for copper decreases. The change in transport for these mutants is potentially interesting as these charged residues may be important for coordinating the copper ion as it passes through the membrane or for proper placement of the copper ion at the extra-cellular domain (E68) and intra-cellular domain (E84) respectively. Given the negative charges of the side chains of these residues, it is possible that copper ions are retarded at these locations in the wild type protein as the ion passes through the membrane. This possibility is supported by our results which show increased rates of transport when these residues are mutated to remove charge and the polar nature of the side chain.

The most dramatic effect is seen with replacements at position H139. When H139 is mutated to an alanine a modest reduction in the Vmax for the transport of copper is seen. No significant effect is seen in the affinity of the protein for copper. However when H139 is mutated to an arginine dramatic changes both in rate of transport and the affinity for copper result. The Vmax for transport increases over 400% with the placement of a positive charge at this position. This is surprising as the histidine is not strictly conserved between yeast and

mammalian Ctr1 proteins (mammalian Ctr1 proteins have a histidine at this position, yeast Ctr proteins do not). However, there is an arginine four amino acids away (~one turn of an α helix) from this histidine in both yCtr1 and yCtr3. One possibility is that a histidine is introduced into the pore formed by the association of perhaps three monomers. Histidine is a titrable side-chain, and not all of these his residues may be protonated simultaneously. Replacement with arginine residues places three fixed positive charges in the pore which may result in charge repulsions widening the pore at a critical site, lessening interactions with the permeating copper ion and increasing the maximal transport rate. The details of the interactions and involvement of his and arg residues in the pathway are the subject of ongoing studies.

Bringing together the results from this study and others we can begin to develop a map of residues that are important for hCtr1 function (Figure 3.7). We have modeled the three trans-membrane domains as α helices and have color-coded the residues that have been suggested to play important roles in copper transport. Residues that when replaced appear to have dramatic effects on the reduction of copper transport are shown in red (M150, M154 G167 and G171). These residues are found on the same face of the α helices of trans-membrane 2 (M150, M154) and three (G167 and G171). The two methionine residues have been suggested to be involved in coordination of copper(Puig, Lee et al. 2002). Aller et al suggest that the two glycine residues are important for helix packing of the trans-membrane domains and loss of complementation in yeast is due to inability of the protein to form a functional unit (Aller, Eng et al. 2004).

Residues that affect copper transport but to a more modest extent have been colored yellow. It is premature to specify what these residues roles are in transport by hCtr1.

However the location of these residues in the three trans-membrane domains is interesting. In trans-membrane 1, Y83 M81 and E84 are all located on one half of the helix in close proximity to where H139 might be in helix 2. These three residues are located at the membrane interface with the intra-cellular portion of the protein and may play a role in regulating copper exit from the translocation pore. Other residues, (Y156, Y147, C161 and E69) are located at the interface of the membrane and extra-cellular space. The two tyrosines are in close proximity to the two methionines that are important for the possible coordination of copper prior to entry into the translocation pore. C161 is implicated as a potential structurally important residue given our present results with the double mutant C161s/C189S and results from others (Aller, Eng et al. 2004). It is unlikely that C161 plays a role in coordinating copper (Eisses and Kaplan 2002). This model supports earlier suggestions that trans-membrane 3 is involved in the interaction of helices in the monomer or interaction of multiple subunits.

Finally, the residue that significantly increases copper transport is labeled green. H139 appears to be important in regulating the affinity of hCtr1 for copper. By placing a positive charge at this position the affinity of hCtr1 is lowered and a dramatic increase in the rate of transport is achieved. Mutations in this study as well as other earlier reports typically cause a decrease in the rate of transport mediated by Ctr1 proteins. Significantly, the charged residues in the transmembrane segments when mutated actually increase the rate of transport by hCtr1. The increases in rate of transport by mutations E68L or E84Q are small. However, the large increase in copper transport by the presence of a stable positive charge at position 139 is significant. The mechanism by which H139 affects transport is not known but is the focus of future work from our lab.

We present results that identify a number of amino acids that are important for the function of hCtr1 as a copper transporter. This work provides evidence of the importance of the amino terminus, carboxyl terminus and selected residues in the trans-membrane domains. We have presented a potential model for the role and the arrangement of the trans-membrane helices by incorporating results from this present work and the work of others (Figure 3.8).

3.6 ACKNOWLEDGEMENTS

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Figure 3.1 Protein alignments of putative trans-membrane domains from select mammalian Ctr1 proteins and yeast Ctr1/3.

A. Trans-membrane 1.

B. Trans-membrane 2.

C. Trans-membrane 3.

Residues of identity are displayed in red. Green residues are conserved residues. Blue residues are semi-conserved.

Figure 3.1


A
hCtr1 ------LVINTAGEMAGAFVAVFLLAMFYEGL----------
Monkey Ctr1 -------VINTAGEMAGAFVAVFLLAMFYEGL----------
Canis Ctr1 --LFSGLVINTAGEMAGAFVAVFLLAMFYEGL----------
mCtr1 NLLFSGLVINTPGEMAGAFVAVFLLAMFYEGLKIAREGLL--
yCtr1 ------------GKAFGIFLLFVVAAFVYKLLLFVSWCLEVH
yCtr3 -------YVVKCGEEDAKSDIEELQG-FYNEPSWKTTLIS--
B
hCtr1 -----PHLLQTVLHIIQVVISYFLMLIFMTYN-
monkey Ctr1 ------HLLQTVLHIIQVVISYFLMLIFMTYN-
Canis Ctr1 -MLSFPHLLQTVLHIIQVVISYFLMLIFMTYN-
mCtr1 ---------QTVLHIIQVVISYFLMLIFMTYN-
yCtr1 -------IIRAFLVFTSTMIIYMLMLATMSFVL
yCtr3 YPTFLDHMIRVTIFVLQWGLSYIIMLLFMYYN-
C
hCtr1 LIFMTYNGYLCIAVAAGAGTGYFLFS---------
monkey Ctr1 -IFMTYNGYLCIAVAAGAGTGYFLFS---------
Canis Ctr1 ------NGYLCIAVAAGAGTGYFLFS---------
mCtr1 ----TYNGYLCIAVAAGAGTGYFLFSW--------
yCtr1 ----SFVLTYVFAVITGLALSEVFFNRCKIAMLK-
yCtr3 -----YNGYIIISCLIGAIVGRFIFCYEPLGSLGA

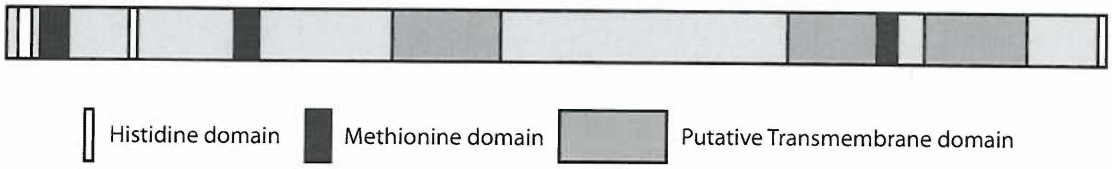
Figure 3.2 Wt hCtr1 expression in stable sf9 cells.

A. Schematic diagram showing putative trans-membrane domains and the relative position of methionine and histidine domains.

B. Western blot showing fractionated membranes for sf9 cells expressing Wt, C189S or cells without any hCtr1 protein. 20 μg of ER, golgi or plasma membrane fractions were loaded onto a 12% SDS PAGE gel. Proteins were visualized using a CT-hCtr1 antibody (1:25,000).

Figure 3.2

A


Histidine domain
Methionine domain
Putative Transmembrane domain

B

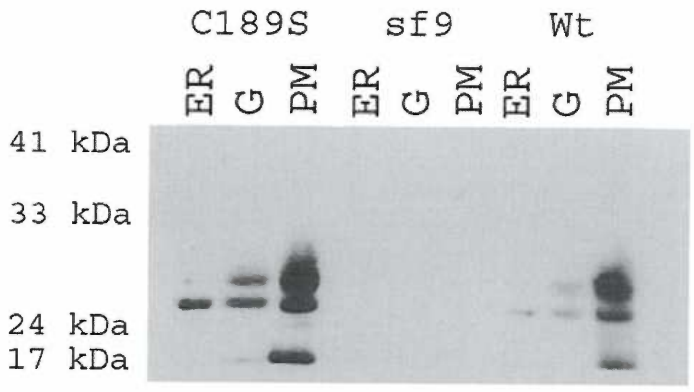

C189S
sf9
Wt
ER
G
PM
ER
G
PM
ER
G
PM
41 kDa
33 kDa
24 kDa
17 kDa

Figure 3.3 hCtr1 truncations.

A. Schematic diagram of hCtr1 with location of truncation mutants displayed.

B. Western blots of N and C terminal truncations expressed in sf9 cells. 20 μg of PM fractions were loaded on 12% SDS PAGE gels. NT truncation proteins were visualized using a CT-hCtr1 antibody (1:25,000). CT truncations were identified using a NT-hCtr1 antibody (1:10,000).

C. Copper uptake curves for NT truncation mutants.

D. Copper uptake curves for CT truncation mutants.

Figure 3.3

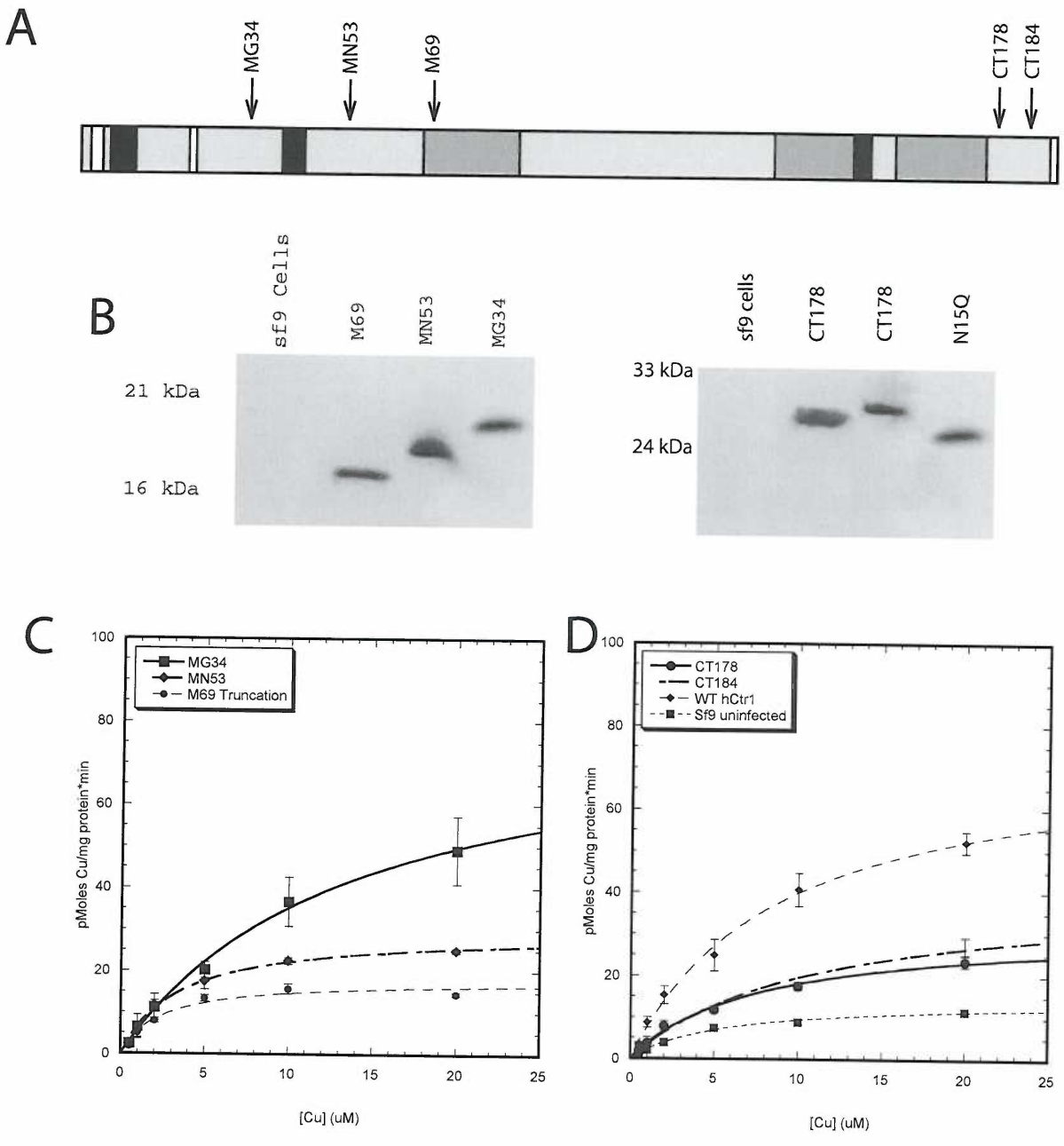

A
MG34
MN53
M69
CT178
CT184
B
sf9 Cells
M69
MN53
MG34
21 kDa
16 kDa
sf9 cells
CT178
CT178
N15Q
33 kDa
24 kDa
C
MG34
MN53
M69 Truncation
pMoles Cu/mg protein*min
[Cu] (uM)
D
CT178
CT184
WT hCtr1
Sf9 uninfected
pMoles Cu/mg protein*min
[Cu] (uM)

Figure 3.4 Methionine mutants of hCtr1.

A. Diagram showing the relative positions of trans-membrane methionine residues in hCtr1.

B. Western blot analysis of methionine mutants. 20 μg of ER, G, or PM membrane fractions loaded on a 12% gel. hCtr1 mutants were identified using CT-hCtr1 antibody (1:25,000).

C. Copper uptake curves for methionine mutants.

Figure 3.4

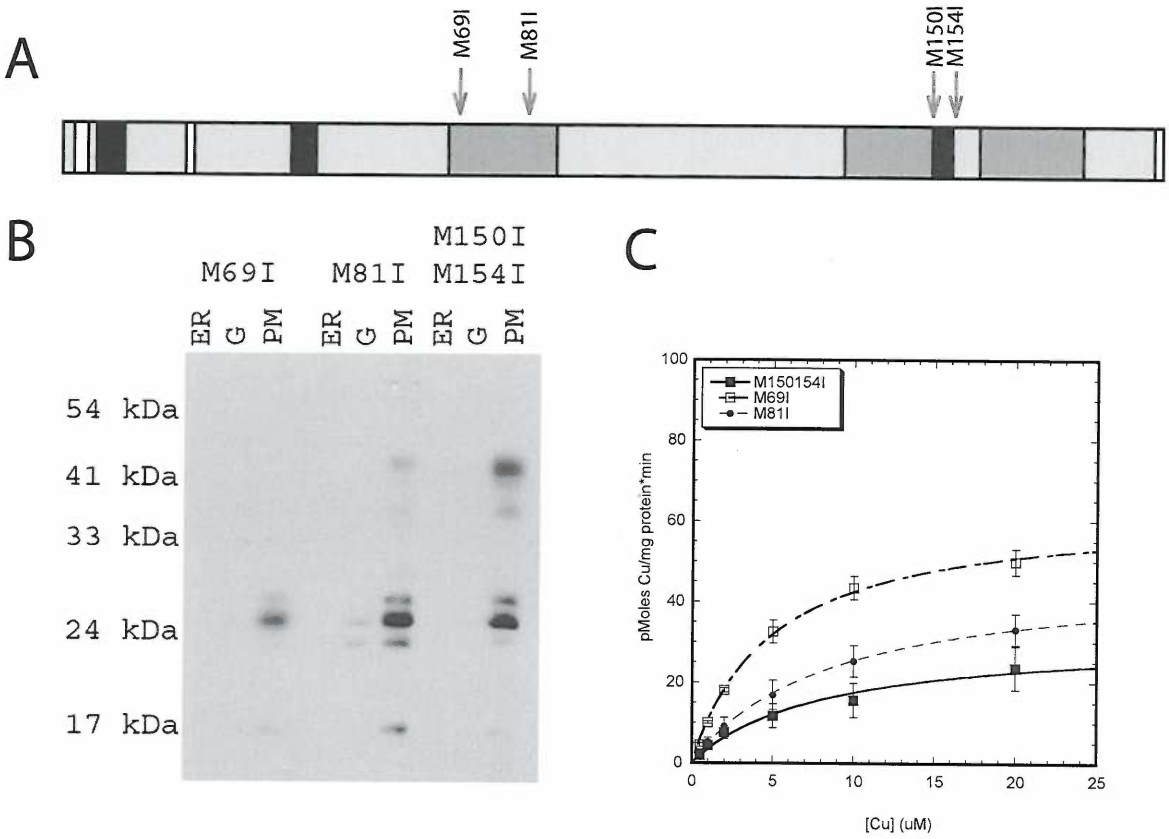

A
M69I
M81I
M150I
M154I
B
M69I
M81I
M150I
M154I
ER
G
PM
ER
G
PM
ER
G
PM
54 kDa
41 kDa
33 kDa
24 kDa
17 kDa
C
M150154I
M69I
M81I
pMoles Cu/mg protein*min
[Cu] (uM)
0
20
40
60
80
100
0
5
10
15
20
25

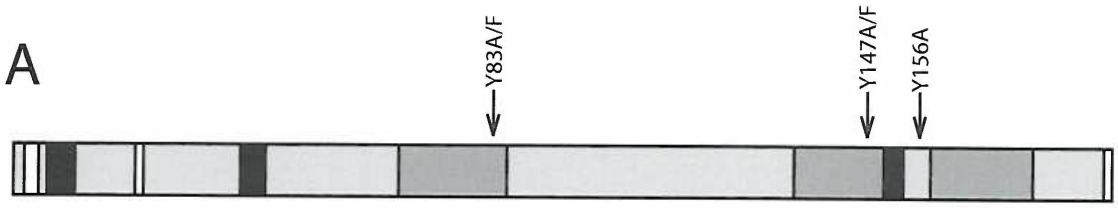
Figure 3.5 Tyrosine mutants of hCtr1.

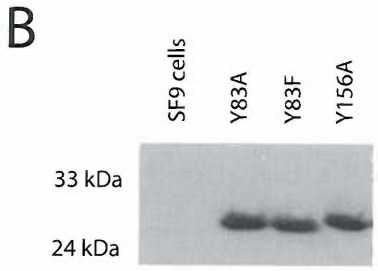
A. Diagram showing the relative positions of trans-membrane tyrosine residues in hCtr1.

B. Western blot analysis of tyrosine mutants. 20 μg of PM membrane fractions loaded on a 12% gel. hCtr1 mutants were identified using CT-hCtr1 antibody (1:25,000).

C. Copper uptake curves for representative tyrosine mutants.

Figure 3.5


A
Y83A/F
Y147A/F
Y156A


B
SF9 cells
Y83A
Y83F
Y156A
33 kDa
24 kDa

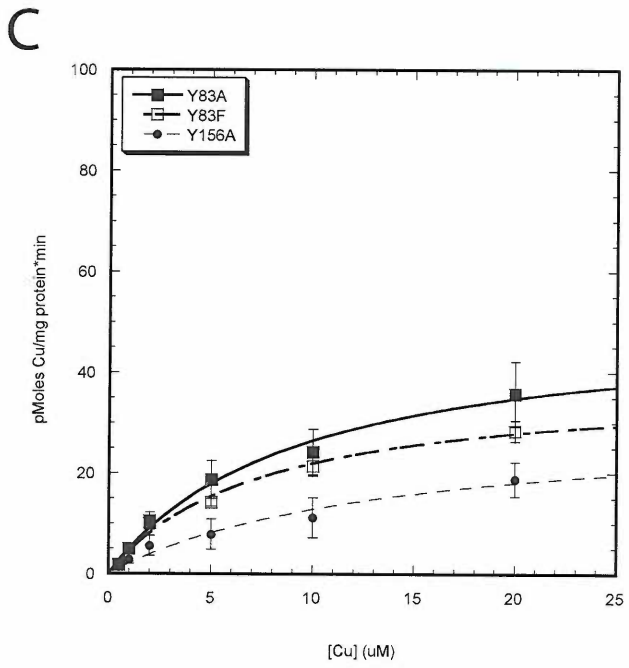

C
Y83A
Y83F
Y156A
pMoles Cu/mg protein*min
[Cu] (uM)
0
20
40
60
80
100
0
5
10
15
20
25

Figure 3.6 Charged residues in trans-membrane domains.

A. Schematic diagram of hCtr1 with location of charged residues displayed.

B. Western blot of E68, E84, H139 and C161S/C189S mutants expressed in sf9 cells. 20 μg of PM fractions were loaded on 12% SDS PAGE gels. Mutants were identified using a CT-hCtr1 antibody (1:25,000).

C. Copper uptake curves for C161S/C189S mutant.

D. Copper uptake curves for charged residues in trans-membrane domains.

Figure 3.6

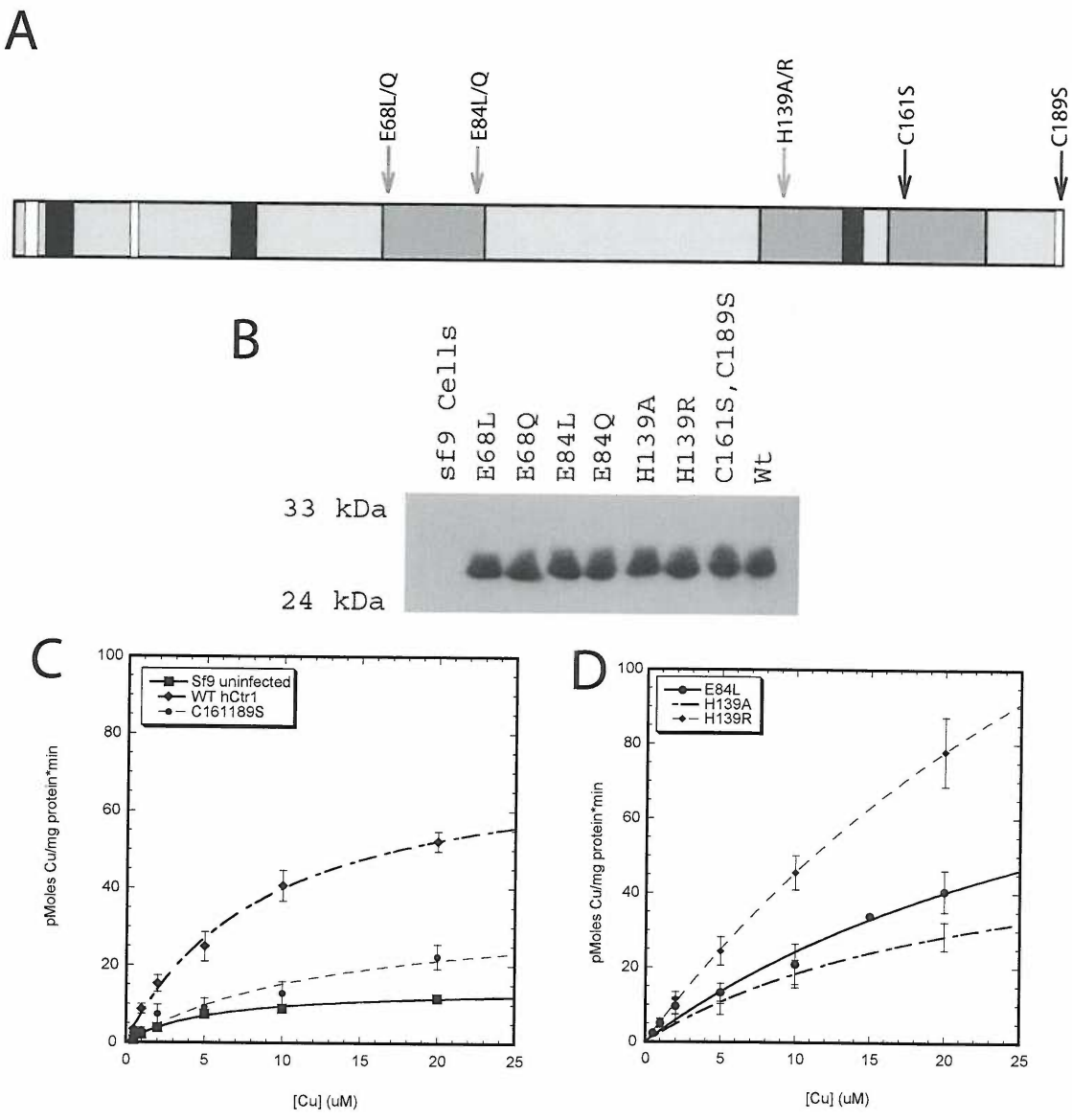

A
E68L/Q
E84L/Q
H139A/R
C161S
C189S
B
sf9 Cells
E68L
E68Q
E84L
E84Q
H139A
H139R
C161S,C189S
Wt
33 kDa
24 kDa
C
Sf9 uninfected
WT hCtr1
C161189S
pMoles Cu/mg protein*min
[Cu] (uM)
D
E84L
H139A
H139R
pMoles Cu/mg protein*min
[Cu] (uM)

Figure 3.7 Putative models of trans-membrane domains of hCtr1.

Putative transmembrane domains are modeled as α helices. Amino Acid residues that are important for copper uptake are shown in color. Mutations that disrupt copper transport significantly are red. Residues that have a moderate effect on transport are in yellow. Residues that stimulate transport are in green.

Figure 3.7

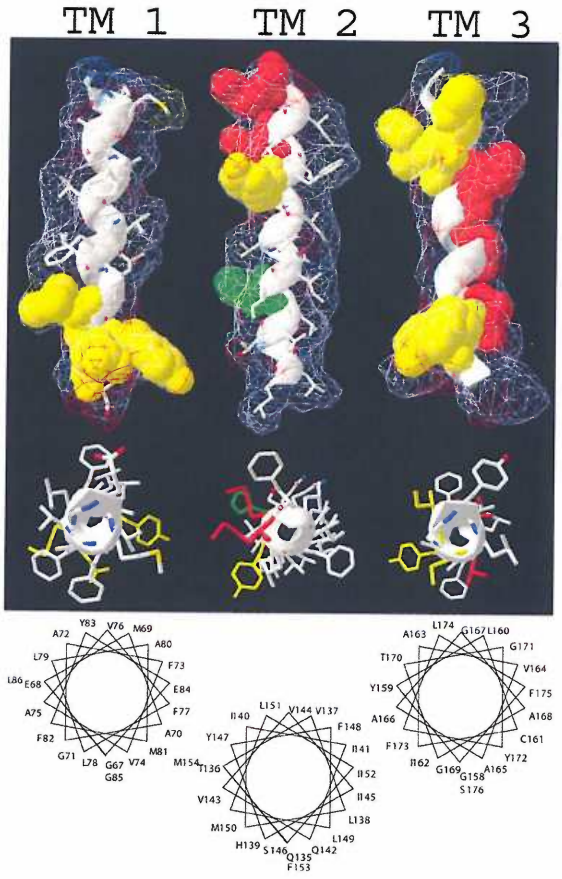

TM 1
TM 2
TM 3
Y83 V76 M69
A72
A80
L79
F73
L80 E68
E84
A75
F77
F82
A70
G71 L78 G67 V74
M81
G85
M154
L151 V144 V137
I140
F148
Y147
I141
T136
I152
V143
I145
M150
L138
H139 S146 Q135 Q142
L149
F153
L174 G167 L160
A163
G171
T170
V164
Y159
F175
A166
A168
F173
C161
I162
Y172
G169 G158 A165
S176

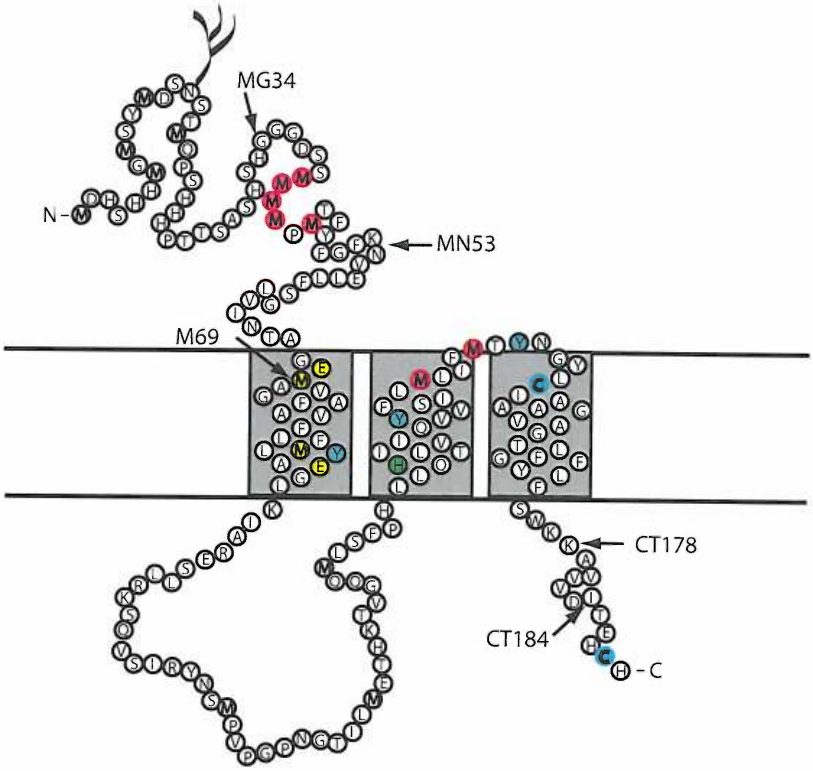
Figure 3.8 Model for copper uptake by hCtr1.

We incorporate information from others as well as the results reported in this study to generate a two dimensional model for the arrangement of hCtr1 complexes in the membrane. **A)** Amino acids that are mutated in this study are shown in the primary sequence of hCtr1. Residues that significantly reduce the transport of copper into cells are shown in red. Residues that have moderate affects on transport are shown in blue and yellow. Residues that increase transport rates are shown in green.

B) A two dimensional model for the position of hCtr1 monomers in the plasma membrane. Only two monomers are shown, however three or four monomers are probably involved in the formation of a functional complex. Residues are shown in color as described in A.

Figure 3.8

A



B

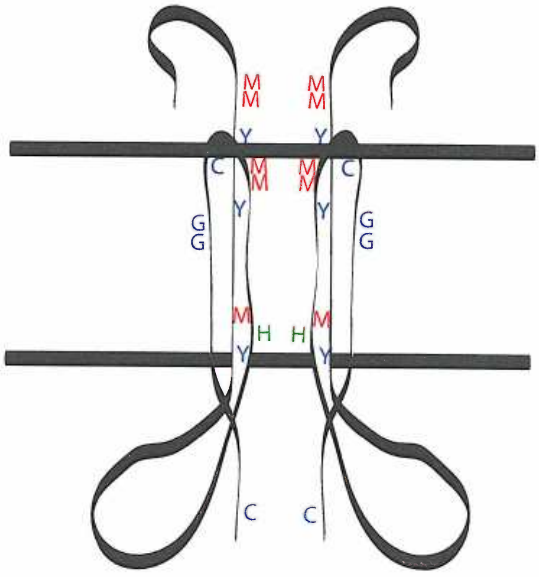


Table 3.1 Mutagenic primers used for generation of mutant constructs of hCtr1.

Forward and reverse primers are shown for each mutation generated. Primers are shown in a 5' to 3' orientation.

Table 3.1 Mutagenic primers

MG34 hCtr1	GAATTCATGGGTGGAGGAGACAGCAGCATG
MN53hCtr1	GAATTCATGAATGTGGAACTACTGTTTTCC
M69I For	CAGCTGGAGAAATCGCTGGAGCTTTTGTGG
M69I Rev	CCACAAAAGCTCCAGCGATTTCTCCAGCTG
M81I For	GTGTTTTTACTAGCAATCTTCTATGAGG
M81I Rev	CCTTCATAGAAGATTGCTAGTAAAAACAC
M150IM154I	GCTACTTCCTCATCCTCATCTTCATCACCTACAACG
M150IM154I	CGTTGTAGGTGATGAAGATGAGGATGAGGAAGTAGC
Y83F For	GCAATGTTCTTTGAAGGACTC
Y83F Rev	GAGTCCTTCAAAGAACATTGC
Y83A For	GCAATGTTCGCTGAAGGACTC
Y83A Rev	GAGTCCTTCAGCGAACATTGC
Y147F For	GGTCATAAGCTTCTTCCTCATGC
Y147F Rev	GCATGAGGAAGAAGCTTATGACC
Y147A For	GGTCATAAGCGCCTTCCTCATGC
Y47A Rev	GCATGAGGAAGGCGCTTATGACC
Y156A For	GCTCATCTTCATGACCGCCAACGGGTACC
Y156A Rev	GGTACCCGTTGGCGGTCATGAAGATGAGC
E68L For	CAATACAGCTGGACTAATGGCTGGAG
E68L Rev	CTCCAGCCATTAGTCCAGCTGTATTG
E68Q For	CAATACAGCTGGACAAATGGCTGGAG
E68Q Rev	CTCCAGCCATTTGTCCAGCTGTATTG
E84L For	GCAATGTTCTATCTAGGACTCAAGATAGC
E84L Rev	GCTATCTTGAGTCCTAGATAGAACATTGC
E84Q For	GCAATGTTCTATCAAGGACTCAAGATAGC
E84Q Rev	GCTATCTTGAGTCCTTGATAGAACATTGC
H139A For	GCAAACAGTGCTGGCCATCATCCAGGTGG
H139A Rev	CCACCTGGATGATGGCCAGCACTGTTTGC
H139R For	GCAAACAGTGCTGCGCATCATCCAGGTGG
H139R Rev	CCACCTGGATGATGCGCAGCACTGTTTGC
CTD184 Rev	TCATAGCATTCGAACACTACCACTGCC
CTK178 Rev	TCATAGCATTCGAATTCTTCCAGCTGAAGAGG

Table 3.2 Apparent Km and Vmax values for hCtr1 proteins.

Data from Cu uptake experiments at various Cu concentrations were analyzed using the Michaelis-Menten equation to determine the Cu concentration at which uptake was half maximal (K_m). K_m values (μM Cu) were derived from multiple experiments (each experiment consisting of triplicate points). The standard error for the K_m values is shown. Vmax values (pMoles Cu/mg protein*min) are shown for each mutant and standard errors are given. The kinetic value for each mutation in hCtr1 represents a minimum of three independent experiments.

A. Wildtype hCtr1 and sf9 cells.

B. hCtr1 truncation mutants

C. Potential copper ligands

D. Charged transmembrane residues

Table 3.2 Km (μM) and Vmax (pMoles Cu/mg protein*min) values for hCtr1 mutants

A. Wildtype hCtr1 and sf9 cells

Cell Line	Km	Vmax
Wt hCtr1	8.9 ± 1.2	75.7 ± 4.8
Sf9	5.6 ± 0.8	14.7 ± 0.8

B. Truncations of hCtr1

Cell Line	Km	Vmax
MG34	14.0± 2.2	84.1 ± 7.1
MN53	3.5 ± 0.4	29.8 ± 1.3
M69	2.4 ± 0.7	18.0 ± 1.5
CT178	7.9 ± 1.3	32.4 ± 2.3
CT184	11.3 ± 2.6	41.6 ± 4.7

C. Potential Copper ligands in hCtr1

Cell Line	Km	Vmax
M150I,M154I	8.3 ± 2.2	31.8 ± 3.9
M69I	4.8 ± 0.4	62.7 ± 1.3
M81I	8.8 ± 0.4	47.7 ± 0.9
C161S,C189S	13.5 ± 6.8	35.6 ± 9.5
Y83A	9.2 ± 1.9	50.8 ± 5.0
Y83F	7.4 ± 1.5	38.2 ± 3.3
Y147A	8.3 ± 2.2	52.2 ± 6.1
Y147F	10.0 ± 2.2	40.6 ± 4.3
Y156A	13.7 ± 3.6	30.4 ± 3.5

D. Charged transmembrane residues in hCtr1

Cell Line	Km	Vmax
E68L	11.7 ± 2.8	106.1 ± 12.8
E68Q	17.3 ± 5.9	92.4 ± 18.1
E84L	23.6 ± 7.2	83.4 ± 14.3
E84Q	16.1 ± 3.8	106.2 ± 14.1
H139A	13.9 ± 3.1	47.3 ± 5.5
H139R	49.7 ± 4.8	271.5 ± 20.2

CHAPTER 4

Stable Plasma Membrane Levels of hCtr1 Mediate Cellular Copper Uptake

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4.1 ABSTRACT

The human Copper transporter 1 (hCtr1), when heterologously over-expressed in insect cells, mediates saturable Cu uptake. In mammalian expression systems, a rapid Cu-dependent internalization of hCtr1 has been reported in cells that over-express epitope-tagged hCtr1 when exposed to Cu in the external medium. This finding led to the suggestion that such internalization may be a step in the hCtr1 transmembrane Cu transport mechanism. We demonstrate that pre-incubation in Cu- containing media of *sf9* cells, stably expressing hCtr1, has no effect on the initial rate of Cu transport. Furthermore, western blot analyses of fractionated *sf9* cell membranes show no evidence of a regulatory Cu-dependent internalization from the plasma membrane. In similar studies on HEK 293 cells, we show that incubation with Cu does not alter the initial rate of Cu uptake mediated by endogenous levels of hCtr1 compared with untreated cells. Confirmation that hCtr1 mediates this transport is provided by specific siRNA-dependent decreases in hCtr1 protein levels and in Cu transport rates. Western blot analysis and confocal microscopy of HEK 293 cells show that the majority of hCtr1 protein is localized at the plasma membrane and upon Cu treatment, no significant internalization is detected. We conclude that internalization of hCtr1 is not a required step in the transport pathway and suggest that oligomeric hCtr1 acts as a conventional transporter providing a permeation pathway for Cu through the membrane and that internalization of endogenous hCtr1 in response to elevated extra-cellular Cu levels, does not play a significant regulatory role in Cu homeostasis.

4.2 INTRODUCTION

Cu is an essential cofactor for many enzymes in eukaryotic cells and in recent years there has been an increasing understanding of the homeostatic mechanisms that are used to regulate cellular Cu content and to deliver Cu to its required sites (Lutsenko and Petris 2003; Petris 2004). Tightly controlled homeostatic mechanisms are required as Cu is an essential metal, however, as with several other trace metals, excessive metal accumulation is severely toxic {Halliwell, 1984 #110;Fridovich, 1978 #108;Waggoner, 1999 #107}. Cu removal from cells is handled by the ATP7 P-type ATPases, Wilsons and Menkes Disease proteins, which utilize ATP hydrolysis to deliver Cu either to the extra-cellular compartment or into the secretory pathway (Petris, Mercer et al. 1996; Lutsenko and Petris 2003).

The protein(s) responsible for Cu uptake have been less well characterized. A major transporter mediating Cu entry into mammalian cells is Ctr1. Ctr1 is functionally related to the Cu uptake systems first identified in yeast about ten years ago (Dancis, Haile et al. 1994; Dancis, Yuan et al. 1994). Ctr1 apparently mediates the uptake of Cu(I) into cells (Eisses and Kaplan 2002; Lee, Pena et al. 2002), it is essential for embryonic development (Kuo, Zhou et al. 2001; Lee, Prohaska et al. 2001) and some progress has been made towards an understanding of its mechanism, largely through elegant complementation studies in yeast (Puig, Lee et al. 2002).

Utilizing epitope-tagged constructs, it was recently shown that Cu exposure of cells that over-expressed tagged hCtr1 molecules at their surface

showed a rapid and complete internalization from the plasma membrane (Petris, Smith et al. 2003; Guo, Smith et al. 2004). This process occurred within 10 minutes of exposure to Cu levels (5μM) that were close to the Km of the transporter. It was suggested that this Cu-dependent internalization might be a part of the transport pathway, rather like the internalization of the Fe-bound transferrin receptor in the process of cellular acquisition of iron (Klomp, Tops et al. 2002; Petris, Smith et al. 2003; Guo, Smith et al. 2004). In addition, it was also reported that such internalization resulted in degradation of hCtr1 and suggested that the internalization-degradation that was Cu-dependent might be an important regulatory pathway that limited Cu uptake under Cu replete conditions (Petris, Smith et al. 2003).

hCtr1 consists of 190 amino acid residues. It has an extra-cellular amino-terminus, an intra-cellular carboxyl-terminus and has three transmembrane segments (Eisses and Kaplan 2002; Klomp, Juijn et al. 2003). It has been proposed that the methionine-rich amino-terminus plays some role in Cu coordination in a functional oligomeric complex (Puig, Lee et al. 2002), but details of the transport mechanism are still the subject of intensive study. During studies on the structure and function of hCtr1 expressed in *sf9* insect cells using baculovirus-mediated infection, we had observed that initial rates of Cu uptake remained linear for at least one hour in the continued presence of extra-cellular Cu, at concentrations ranging from 2μM to 25μM (Eisses and Kaplan 2002). Likewise, others have seen linear uptake for at least 1 hr in HEK 293 cells over-expressing epitope tagged hCtr1 (Lee, Pena et al. 2002). From our studies in

insect cells, it seemed unlikely that a large fraction of hCtr1 was rapidly internalized from the plasma membrane. If this had occurred, the initial rate of Cu uptake would have been expected to fall.

We report here an investigation of this issue in stably transfected insect cells, and observe no decrease in the functional activity of hCtr1 following exposure to extra-cellular Cu. We also extend this approach to endogenous hCtr1 in HEK 293 cells and in this human cell line we do not observe a change in transport activity of endogenous hCtr1 following incubation with Cu, or a Cu-dependent internalization from the plasma membrane.

4.3 MATERIALS AND METHODS

4.3.1 *hCtr1 Detection*

An antibody against the C-terminal 15 amino acids of hCtr1 was raised in rabbits by Affinity BioReagents, Golden CO. The peptide SWKKAVVVDITEHCH was synthesized and conjugated to KLH. Rabbits were immunized. The resulting antibody was affinity purified using the peptide that it was raised against. Endogenous or over-expressed hCtr1 protein was probed with the hCTR1 antibody at a dilution of 1:50,000.

4.3.2 *hCtr1 expression in insect cells*

hCtr1 constructs were cloned into pIB/V5-HisTOPO vector (Invitrogen, Carlsbad, Ca.). Wt hCtr1 (WT) and C189S cDNA were PCR amplified from a baculovirus expression vector (Eisses and Kaplan 2002) using primers (5' forward primer: 5'-gaattcatggatcattccc-3', and 3' reverse primer: 5'-ccgcggaacaacttcccactgc-3') that contain EcoRI and SacII restriction sites engineered at the 5' and 3' ends respectively. C189S is a mutant of hCtr1 that has the cysteine at amino acid position 189 mutated to a serine residue and migrates primarily as a monomer, as opposed to multimeric molecules (Eisses and Kaplan 2002). The amplified cDNA constructs were ligated into the pIB/V5-HisTOPO vector. The expression plasmids were sequenced to confirm correct cDNA sequence for each construct and then were transfected into *sf9* cells using Cellfectin transfection reagent according to manufacturer's protocol (Invitrogen).

sf9 cells containing integrated hCtr1 cDNA were selected using Blasticidin S (Invitrogen, Carlsbad, Ca.) for two weeks. Expression of the

respective hCtr1 construct was compared to baculovirus-expressed protein and confirmed by western analysis using our anti-hCtr1 antibody. Cells were maintained in Ex-Cell 420 media (JRH Biosciences, Lenexa, KS) containing 0.015 mg/ml Blasticidin S. Cu uptake into cells was measured in each of these cell lines using Cu-64, as previously reported (Eisses and Kaplan 2002), a minimum of three determinations of the rate of Cu uptake were performed for each construct or experiment.

4.3.3 *RNAi suppression*

Two methods were utilized to generate siRNA molecules to use in RNAi analysis in HEK 293 cells. The first method used oligonucleotides selected and synthesized by Invitrogen (Stealth RNAi). The second method uses Invitrogen Block-It RNAi Kit (Invitrogen, Carlsbad, Ca.). Two Stealth RNAi oligonucleotides were tested for knockdown of endogenous hCtr1 protein. The second method involved amplification of hCtr1 and modification of this amplified product using the components of the Block-iT RNAi TOPO Transcription Kit and the Block-iT Dicer RNAi Kit. Briefly, hCtr1 cDNA was amplified using two primers (5'-gaattcatggatcattccc-3' and 5'-aagcttaacaacttcccactgc-3') and T7 Linkers were ligated using the TOPO ligation technology. Equal amounts of sense and anti-sense ssRNA transcripts were annealed to generate dsRNA complexes that were then cut to create 20-25 bp fragments by the Dicer enzyme. These fragments were transfected into HEK 293 cells and analyzed to determine if gene knockdown had occurred. Transfection efficiency was monitored using a

fluorescent oligonucleotide (BLOCK-iT Fluorescent oligo, Invitrogen) and estimated to be 80-90%.

Both sets of RNAi molecules were transfected individually into HEK 293 cells using Lipofectamine 2000 following Invitrogen's protocols. The ability of the RNAi molecules to knockdown hCtr1 expression was analyzed by hCtr1 protein detection using anti-hCtr1 antibody on whole cell extracts and by measuring Copper-64 transport in HEK 293 cells transfected with and without RNAi molecules. As a control for nonspecific knockdown, RNAi were generated from a Lac Z cDNA using the Block-It RNAi Kit.

4.3.4 *Cell fractionation*

Fractionation of insect cells or HEK 293 cells were carried out using a 5 step sucrose step gradient as previously described (Eisses and Kaplan 2002) or using linear Optiprep gradients (Sigma, St. Louis, MO). Briefly, cells were treated with cycloheximide (100μg/ml) for 20 min. followed by +/- 100μM $CuCl_2$ treatment for 2 hours. Cells were pelleted by centrifugation and washed twice with PBS and resuspended in homogenization buffer (0.25 M sucrose, 1mM EDTA 10 mM Hepes-NaOH, pH7.4). Cells were lysed using a dounce homogenizer (20 strokes) and the post nuclear fraction was layered on top of a 5 ml linear gradient. The gradient was centrifuged at 200,000 xg for 3 hours and collected in 0.5 ml fractions by tube puncture. 30μg of protein from each fraction was analyzed by SDS PAGE. The subsequent protein blot was analyzed using the hCtr1 antibody (1:50,000) and an HRP-conjugated Goat anti-Rabbit secondary antibody (1:10,000).

4.3.5 *Immunofluorescence*

HEK 293 cells were grown in 12 well trays for 24-48 hrs on sterile glass cover slips. In each experiment, $CuCl_2$ (100μM) was added to the media of some wells for 2 hrs, while other wells containing HEK 293 cells had no extra copper added. Cells were fixed and permeabilized by the addition of ice-cold acetone followed by a PBS wash. The cells were blocked in PBS containing 1% BSA, 1% gelatin overnight. The cells were probed with a primary antibody at the stated dilution for 1 hr followed by PBS washes. The cells were then probed with secondary antibodies (1:2000) for 1hr followed by PBS washes. Samples were mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, Ca).Immunofluorescence microscopy was performed on a Zeiss LSM510 confocal microscope (Carl Zeiss, Gottingen, Germany).

4.4 RESULTS

4.4.1 *hCtr1 expressed in Insect Cells.*

We have previously shown that viral expression of hCtr1 in *sf9* insect cells provides good yields of functional protein (Eisses and Kaplan 2002). In the present study, we have developed and used two stable *sf9* cell lines expressing hCtr1. Stable cell lines allow more consistent expression of hCtr1 mutant proteins by removing some of the potential variables associated with a lytic viral expression system. Two cell lines were utilized in this study, one expressing a WT construct and a second expressing a single cysteine substitution mutant, C189S.

We tested the ability of cells expressing these two constructs to transport Cu across the plasma membrane in *sf9* cells. The cell line expressing the WT transporter showed saturable Cu uptake with an apparent Km of 8.2 ± 0.8 μM and a Vmax of 84 ± 3.6 pMoles Cu/mg protein/min. Similarly, C189S had an apparent Km of 6.8 ± 0.6 μM and a Vmax of 66 ± 2.3 pMoles Cu/ mg protein/min (data not shown). The Km values for these two constructs are similar to those reported earlier using the baculovirus-mediated expression system (Eisses and Kaplan 2002).

Uptake experiments were performed using a copper concentration of 5 μM assayed for 65 min. We observed linear uptake of Cu-64 in both cell lines for at least 1 hour. Cu-64 uptake was 3.5 fold greater in hCtr1 cell lines than in *sf9* cells not expressing hCtr1. There was no demonstrable decrease in the rate of uptake during the 65 min measurement (Figure 4.1).

Recently, reports suggesting rapid internalization of hCtr1 by extra-cellular copper have speculated about the role of this regulation on the transport of Cu into cells (Petris, Smith et al. 2003). It has been reported that hCtr1 is internalized at copper levels similar to the apparent Km of hCtr1 for Cu transport. This led to the suggestion that binding of Cu to hCtr1 induces internalization with Cu bound. A subsequent event occurs once the Cu-bound hCtr1 is internalized that allows Cu to be transported from inside the endosome (or some other compartment) to proteins in the cytoplasm of cells either via hCtr1 or another as yet unidentified protein. It was postulated that this mechanism might provide the basis for Cu uptake into cells (Klomp, Tops et al. 2002; Petris, Smith et al. 2003).

This model for Cu transport is reminiscent of transferrin receptor-mediated uptake of iron. We have tested this proposal in insect cells expressing C189S hCtr1 and examined the impact of exposure to extra-cellular copper on hCtr1 proteins at the plasma membrane. C189S was selected as it is predominantly monomeric in SDS gels(Eisses and Kaplan 2002). Cells stably expressing C189S were pre-treated for 10 min with 100 μM $CuCl_2$. The cells were washed with transport buffer and the rate of uptake into these cells was compared with cells that had not been exposed to Cu. The results are shown in Figure 4.1. Clearly the pre-exposure to Cu is without effect on the kinetics of Cu uptake. Internalization of a significant fraction of hCtr1 at these high Cu levels would result in a decrease in the rate of Cu uptake. Similarly, western blot analysis of C189S cells pretreated or not with 100 μM Cu showed no significant difference in the intensity of the hCtr1 C189S protein in the plasma membrane

(Figure 4.1, inset). This provides strong evidence that significant internalization of hCtr1 is not a necessary step in the transport mechanism. Consequently, there is no reason to suppose that hCtr1 mediates transport by a mechanism other than a conventional transport pathway.

4.4.2 *Endogenous hCTR1 expression in HEK293 cells*

Previous studies that have described Cu-dependent internalization have been carried out in mammalian cells that have been engineered to over-express an epitope-tagged version of hCtr1. We decided to extend our experiments to assess the role that internalization might play in regulation of endogenous hCtr1 levels at the plasma membrane of HEK 293 cells. Utilizing a similar strategy as we described above in insect cells, we measured the rate of isotopic Cu uptake into HEK293 cells following their incubation in the presence of 50 μM Cu (data not shown), 100 μM Cu, or in the absence of Cu. There was no impact of Cu pre-treatment on the uptake of extra-cellular Cu (Figure 4.2), where uptake was linear for at least 80 minutes following Cu pre-treatment, and western blot analysis of plasma membrane fractions isolated from these two sets of cells showed no significant change in the amount of hCtr1 protein (Figure 4.2, inset). To better assess the possibility of shifts of hCtr1 from the plasma membrane to internal locations within the cell, we fractionated cells on a linear gradient after cycloheximide treatment followed by 3 hr copper treatment with 100 μM $CuCl_2$. There is no significant movement of the hCtr1 from the plasma membrane to internal fractions either in cells pretreated with 100 μM $CuCl_2$ or cells receiving no Cu pretreatment (data not shown).

One question that arises from the experiments with HEK293 cells is whether or not the major fraction of Cu uptake measured is mediated by endogenous hCtr1, or by some other transporter (see Discussion). In order to examine this issue, we utilized an RNAi approach. We treated HEK293 cells with specific oligonucleotides designed to reduce hCtr1 protein levels. We examined both the rate of uptake of Cu into cells following RNAi knockdown, as well as the antibody signal reflecting protein levels of hCtr1 in the plasma membrane. There is a significant decrease in hCtr1 expression in cells that have been treated with siRNA compared with control cells (Figure 4.3, inset). Similarly, there is an approximately 80% decrease in the rate of Cu uptake into the treated cells. This confirms that the major fraction of the Cu uptake into HEK293 cells is mediated by hCtr1 (Figure 4.3). These experiments demonstrate that endogenous hCtr1 protein is the primary protein involved in the Cu uptake pathway into these mammalian cells. Significantly, hCtr1 is available to mediate Cu uptake in both replete and Cu-depleted extra-cellular conditions. We do not see any shift of endogenous hCtr1 protein from the plasma membrane nor do we see any loss of total hCtr1 protein (nor significant degradation) from the cellular pools of these cells.

4.4.3 *Confocal microscopy of endogenous hCtr1 cellular protein.*

Recent studies investigating the cellular localization of hCtr1 using confocal microscopy have produced conflicting results. The primary location of hCtr1 protein within the cell has varied depending on which cell line was examined (Klomp, Tops et al. 2002; Petris, Smith et al. 2003). Most of this work

has been carried out utilizing over-expressed epitope-tagged protein. In order to complement our characterization of the functional properties of endogenous hCtr1 protein, we utilized confocal microscopy to image the hCtr1 protein pools in HEK293 cells. Using our hCTR1 antibody (1:500), we probed the cellular location of endogenous hCtr1 in HEK293 cells. As shown in Figure 4.4, we see a significant amount of antibody staining at the plasma membrane. We next examined the effects that excess Cu in the extra-cellular medium might have on the amount and location of endogenous hCtr1 protein. HEK293 cells were plated on cover slips and allowed to grow until 50% confluent. The cells were then either treated with 100 μM $CuCl_2$ or were left in media containing no excess Cu. The results of these experiments are shown in Figure 4.4 (top two panels). We see endogenous hCtr1 protein at the plasma membrane in both Cu treated cells and in cells with no Cu pretreatment. It is difficult to accurately quantitate the levels of protein found at the plasma membrane but it is clear that a significant portion remains at the plasma membrane, competent to mediate Cu uptake.

To confirm that the hCtr1 staining we see is at the plasma membrane, we double labeled HEK293 cells with our hCTR1 antibody as well as with an antibody raised against the β-subunit of the Na,K ATPase (1:500). The latter protein is not sensitive to extra-cellular Cu concentration. As can be seen in Figure 4.4 G and H, Cu pretreatment has little affect on the localization of either protein and co-localization of these two proteins occurs at the plasma membrane.

4.5 DISCUSSION

The absence of a Cu-dependent decrease in the rate of Cu uptake and no loss of hCtr1 from the plasma membrane of insect cells confirm that internalization of Cu-bound transporter is not an essential step for Cu uptake mediated by hCtr1. It seems likely that hCtr1 mediates Cu uptake in a more conventional manner. We suggest that hCtr1 provides a Cu permeation pathway through the plasma membrane. There have been several studies since the first observations on the function of hCtr1 that have concluded that the 28kD protein probably forms oligomers in the plasma membrane (Pena, Puig et al. 2000; Eisses and Kaplan 2002; Lee, Pena et al. 2002; Aller, Eng et al. 2004). It seems likely that a stable oligomer of hCtr1 monomers forms in the cell membrane and this complex mediates the uptake of Cu into the cell. It has been suggested that these oligomers are probably trimeric, although other structures, tetramers or trimers of dimers have not been ruled out. We suggest that the important methionine-rich domains in the extra-cellular amino-terminus (Eisses and Kaplan 2002; Puig, Lee et al. 2002; Klomp, Juijn et al. 2003) provide a Cu-coordinating center that localizes Cu to the mouth of the trans-membrane transport pathway. hCtr1 remains at the plasma membrane and mediates this metal transport pathway. We are currently investigating the details of this proposal utilizing site-directed mutagenesis and functional transport assays.

The suggestion that elevated extra-cellular Cu might directly feed-back to reduce its own cellular uptake is attractive, especially in the light of post-translational down-regulation of Cu transporters that has been observed in yeast

following their internalization (Ooi, Rabinovich et al. 1996). The initial report of Cu-dependent internalization of epitope-tagged hCTR1 in HEK293 cells occurred at Cu levels as low as 2 μM and was essentially complete in 10 minutes (Petris, Smith et al. 2003).

Subsequent studies supported this observation in HEK293 cells at elevated Cu levels of around 50μM to 100μM. Although both labeling and confocal microscopic evidence was presented for these epitope-tagged constructs, it should be emphasized that no functional corollary was demonstrated. Studies in other cells (HeLa and Caco2 cells) did not observe such Cu-dependent relocalization of endogenous levels of hCTR1 (Klomp, Tops et al. 2002), and these authors concluded that these putative regulatory phenomena may be cell-specific. It should be borne in mind that although hCtr1 expression is apparently essential for embryonic development and Cu uptake is its sole known physiological function, it is not the only transporter that can mediate Cu uptake. It has been suggested that in some intestinal cells Cu uptake is mediated by the divalent metal ion transporter, DMT1 (Arredondo, Munoz et al. 2003). In the present work we have shown that in HEK 293 cells, a human cell line, hCTR1 is responsible for at least 80% of the Cu uptake. It is interesting that in intestinal cells it has been reported that Cu uptake is stimulated (and not decreased) by exposure to elevated Cu levels (Zerounian, Redekosky et al. 2003; Arredondo, Cambiazo et al. 2004). It has yet to be shown which of the Cu uptake proteins mediates the uptake pathway in these intestinal cells.

In the present work we supply functional (Cu uptake measurements), biochemical (cell fractionation), RNA knockdown and confocal microscopic data that together suggest that endogenous levels of hCTR1 are stably expressed in the plasma membrane and remain functional in the face of elevated extra-cellular Cu. We suggest that Cu uptake is mediated by hCTR1 by providing a transport pathway across the plasma membrane and that the regulation of cellular Cu content in the face of elevated extra-cellular Cu levels more likely occurs via regulation of the Cu exit pathways. Such regulation of exit pathways has been reported previously (Petris, Mercer et al. 1996) to occur in the re-localization of ATP7A, the Menkes disease protein from intracellular locations to the plasma membrane in response to elevated Cu levels.

4.6 Acknowledgements.

We are grateful to Dr Natalie Barnes for advice on the confocal microscopy work. The production of Cu-64 at Washington University School of Medicine is supported by the NCI grant R24 CA86307. This work was supported by NIH Grant P01 GM067166, Copper Entry into Human Cells, Project #1 to JHK.

Figure 4.1 Copper Uptake Time Course in *sf9* Cells.

Copper uptake experiments were carried out on *sf9* cells expressing C189S (■) or *sf9* cells alone (●) to assess the effect of extra-cellular Cu. Cells were either treated with 100 μM $CuCl_2$ (closed symbols) for 10 minutes prior to uptake experiments or left untreated (open symbols). The results shown are typical of those obtained in three independent experiments. **Inset**: western analysis of cells expressing C189S (2&4) or *sf9* cells alone (1&3). Cells were pretreated with copper (100 μM $CuCl_2$, lanes 3&4) or no copper (lanes1&2).

Figure 4.1

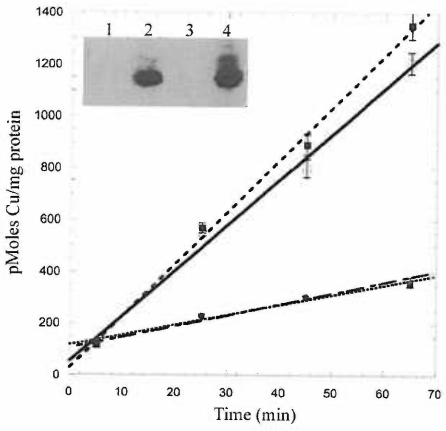

1400
1200
1000
800
600
400
200
0
pMoles Cu/mg protein
0
10
20
30
40
50
60
70
Time (min)
1
2
3
4

Figure 4.2 Copper Pre-treatment of HEK293 Cells.

HEK293 cells were either pretreated with 100μM $CuCl_2$ (■) or no Cu (●) for 10 minutes prior to uptake experiments. Cells were plated in 12 well culture dishes and allowed to grow to 80-90% confluence. The results shown are typical of results obtained from four independent experiments. **Inset**: 40ug of total membranes were run on a 10% SDS PAGE gel and blotted to nitrocellulose membrane. The membrane was probed with anti-hCtr1 (1:50,000). Lanes 1and 2 represent HEK293 cells without Cu pretreatment, Lanes 3 and 4 represent HEK293 cells with Cu pretreatment (100 μg/ml cycloheximide for 20 minutes, 100 μM $CuCl_2$, 2 hours).

Figure 4.2

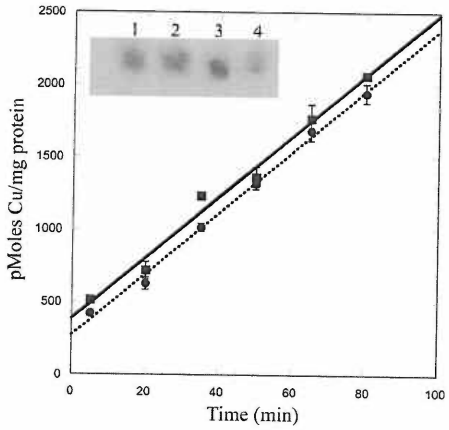

1
2
3
4
2500
2000
1500
1000
500
0
pMoles Cu/mg protein
0
20
40
60
80
100
Time (min)

Figure 4.3 RNAi analysis of hCtr1 in HEK293 cells.

siRNA was transfected into HEK293 cells grown in 12 well culture plates. Copper uptake was measured on HEK 293 cells alone (▲) or cells transfected with siRNA from control Lac Z cDNA (♦), Stealth hCtr1siRNA (●), or diced hCtr1 siRNA (■). Results shown are similar to those results obtained in three independent experiments. **Inset**: Western analysis of HEK293 cells transfected with RNAi. 30 μg of total membranes were loaded on 10% SDS PAGE gel from cells used in RNAi uptake experiments (lane 1: HEK293 cells, lane 2: Control siRNA cells, lane 3: diced hCtr1 siRNA cells, and lane 4: stealth hCtr1 siRNA cells). Proteins were blotted to nitrocellulose and probed with anti-hCtr1 antibody (1: 50,000).

Figure 4.3

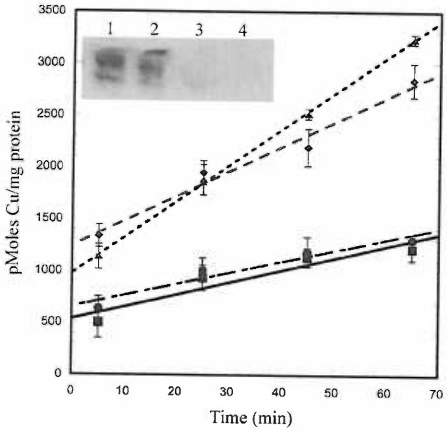
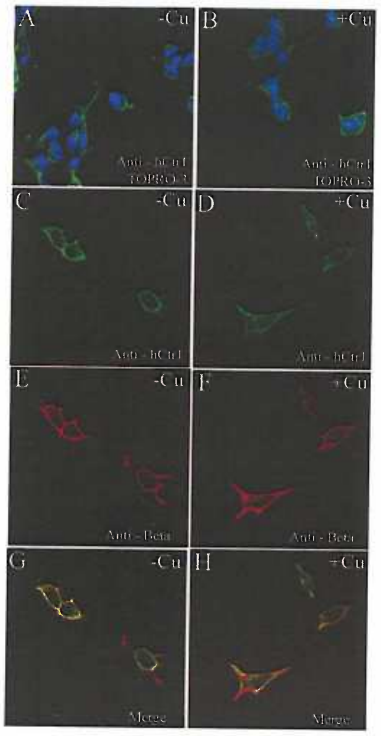

1
2
3
4
3500
3000
2500
2000
1500
1000
500
0
pMoles Cu/mg protein
0
10
20
30
40
50
60
70
Time (min)

Figure 4.4 Localization of endogenous hCtr1 in HEK293 cells.

HEK293 cells were fixed and permeabilized by incubation in acetone. Cells were probed with primary antibodies directed against hCtr1 (A-D, 1: 500 dilution) followed by an Alexa 488 goat anti-rabbit antibody (1: 2000 dilution, Molecular Probes, Eugene, Or.). HEK293 cells were also labeled with TO-PRO-3 iodine (A&B, 1: 10,000 dilution, Molecular Probes) or an antibody against the β–subunit of the Na,K ATPase (E&F, 1:500 dilution, Affinity BioReagents, Golden, Co.) followed by a Cy5 donkey anti-mouse antibody (1: 800 dilution, Jackson ImmunoLabs, West Grove, PA.). Panels G&H are a merge of panel C & E (G) and D&F (H) with overlap staining patterns seen as yellow staining. The Cy5 color (blue) has been changed to red to allow easier visualization of the overlap.

Figure 4.4


A
-Cu
Anti - hCtr1
TOPRO-3
B
+Cu
Anti - hCtr1
TOPRO-3
C
-Cu
Anti - hCtr1
D
+Cu
Anti - hCtr1
E
-Cu
Anti - Beta
F
+Cu
Anti - Beta
G
-Cu
Merge
H
+Cu
Merge

Chapter 5

Differential Regulation by Copper of Endogenous and Over-expressed hCtr1 in Mammalian Cells

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5.1 ABSTRACT

Regulation of copper import and export is an important process demonstrated by mutations in copper export proteins. Menkes disease is a lethal condition that is caused by the loss of copper transport in the small intestine. Regulation of import and export proteins maintains adequate copper levels within the cell without toxic side effects. Recently it was reported that hCtr1 undergoes rapid and complete loss of protein from the plasma membrane in response to increases in extracellular copper (Petris, Smith et al. 2003). hCtr1 is believed to be a plasma membrane copper transporter. Rapid internalization of hCtr1 protein in response to copper concentrations near hCtr1's apparent Km has led to the suggestion that hCtr1 may act more as a receptor, binding copper and then internalizing to an endocytic compartment where copper is transported and released (Petris, Smith et al. 2003; Guo, Smith et al. 2004).

We investigated the differences between copper-dependent regulation of over-expressed hCtr1 and the lack of this regulation of endogenous protein. We could detect no internalization of endogenous hCtr1 analyzed by several different biochemical techniques. hCtr1 was biotinylated at lysine 52 and cell surface biotinylation indicated that endogenous hCtr1 lacked any copper-mediated regulation. However, we were able to detect internalization of epitope-tagged, over-expressed hCtr1 protein using these same techniques. Additionally, we can recapitulate experiments done by others that allow hCtr1 to be rapidly internalized to an intracellular location. We discuss how this difference might occur and underscore the importance of the choice of expression systems for experimental design. These data suggest that hCtr1 is a functional transporter at the plasma membrane and likely acts as a permeation pathway through the plasma

membrane.

5.2 INTRODUCTION

The proteins of yeast that are involved in copper homeostasis are regulated so that adequate but not excessive copper levels are maintained. (Jungmann, Reins et al. 1993; Pena, Koch et al. 1998{Dancis, 1994 #607);(Dancis, Yuan et al. 1994). When copper is depleted in yeast cells, transcription factors turn on uptake genes, namely the copper transporter proteins yCtr1 and yCtr3, to increase cellular copper levels (Dancis, Haile et al. 1994). Under copper-replete conditions, transcription factors work in concert to turn off copper uptake and increase mechanisms for copper buffering and export (Furst, Hu et al. 1988; Dancis, Haile et al. 1994). In addition post-transcriptional regulatory degradation aids in further lowering the levels of membrane transporters mediating copper uptake (Ooi, Rabinovich et al. 1996; Yonkovich, McKenndry et al. 2002). Some of the mechanisms for the regulation of copper export are present in mammalian cells (reviewed in(Lutsenko and Petris 2003)), however no transcriptional regulation has so far been identified. Recently, the issue of regulatory control of copper uptake in mammalian cells has been the subject of investigation. There are conflicting reports about how the human copper transporter 1 protein (hCtr1) is regulated in response to variations in copper levels in the extracellular milieu (Klomp, Tops et al. 2002; Petris, Smith et al. 2003; Eisses, Chi et al. 2005). When extracellular copper levels are raised, it has been reported that,in HEK 293 cells, the plasma membrane level of hCtr1 falls as the transporter is internalized (Petris, Smith et al. 2003; Guo, Smith et al. 2004), while others have either reported that such internal localization is cell-specific (Klomp, Tops et al. 2002), or have failed to observe significant decreases of plasma membrane hCtr1, in HEK 293 cells in response to elevated extracelleular copper (Eisses, Chi et al. 2005).

In yeast, Ctr1 transcription is regulated by copper and yCtr1 protein is also regulated in a post translational manner (Ooi, Rabinovich et al. 1996; Yonkovich, McKenndry et al. 2002). Ctr3 regulation is predominately at the transcriptional level. yCtr1 is recycled into intracellular vesicles in the presence of elevated copper levels. Additionally, yCtr1 can be degraded at the plasma membrane in an endocytosis-independent manner regulated by the transcription factor Mac1 (Ooi, Rabinovich et al. 1996; Yonkovich, McKenndry et al. 2002). This copper- dependent post translational regulation of Ctr1 protein at the plasma membrane led others to investigate the possibility that Ctr1 might also be regulated in a similar manner in mammalian cells.

Klomp et al examined hCtr1 expression in a number of mammalian cell lines (Klomp, Tops et al. 2002). They observed that the localization of endogenous hCtr1 was specific to a particular cell type. In a number of cell types (Caco-2, HEK 293 and CHO), hCtr1 was localized at the plasma membrane, as expected for a protein that has been shown to mediate copper uptake into cells (Eisses and Kaplan 2002; Lee, Pena et al. 2002). Surprisingly, in other cell types (HeLa, A549, H441 and BeWo), hCtr1 exhibited an intracellular localization (Klomp, Tops et al. 2002). Klomp et al incubated a variety of cells in media with high or low (in the presence of the copper chelator BCS) levels of extracellular copper but saw no change in hCtr1 location under these conditions. When basal endocytosis was inhibited by the addition of methyl-β-cyclodextran (MβCD), in cell types that had a predominantly plasma membrane location, hCtr1 was stabilized at the plasma membrane. This suggests that hCtr1 may relocalize from the plasma membrane to intracellular compartments, but in a copper-independent manner.

Petris et al have presented data that suggested that c-myc epitope tagged, over-expressed hCtr1 is regulated in a copper dependant manner in HEK 293 cells (Petris, Smith et al. 2003). When incubated with high levels of copper (100μM), hCtr1 disappeared from the plasma membrane and was localized to an intracellular compartment. Surprisingly, hCtr1 was also rapidly internalized and degraded in the presence of modest copper levels. It was reported that with 2-5 μM extracellular copper, there is a rapid internalization (within 10 minutes) of hCtr1 and subsequent degradation of the protein in the presence of protein synthesis inhibitors (Petris, Smith et al. 2003). It was subsequently reported that only functional proteins undergo this regulation, as proteins defective in copper uptake (M150, M154, and M45 mutants) do not undergo this type of regulation (Guo, Smith et al. 2004). This led to the suggestion that the methionine clusters play a role in facilitating the copper dependent regulation suggested for hCtr1. It was also suggested that internalization of hCtr1 may be an essential step in the copper uptake mediated by hCtr1. In a similar fashion to transferrin-mediated uptake of iron, their model suggests that hCtr1 binds copper in the extracellular space and this binding triggers the internalization of the copper-hCtr1 complex. Release of copper and transport across an intracellular membrane then occurs inside the cells either mediated by hCtr1 or another unidentified protein in vesicles (Petris, Smith et al. 2003; Guo, Smith et al. 2004).

Recently, we reported that copper uptake mediated by hCtr1, heterologously expressed in insect cells, occurs in the absence of internalization, so that internalization cannot be a necessary step in copper uptake mediated by hCtr1. Furthermore, endogenous hCtr1 remains at the plasma membrane and is not regulated by increases in extracellular

copper in HEK 293 cells (Eisses, Chi et al. 2005). We showed that endogenous hCtr1 protein appears to be stable at the plasma membrane for 2 hours after exposure to high (100 μM) extracellular copper levels in HEK 293 cells, and that under these conditions the rate of copper uptake remains linear with time (Eisses, Chi et al. 2005).

In this present study, we have examined in detail the effects of copper on the cellular location of hCtr1. Utilizing several complementary techniques, cell surface biotinylation, confocal microscopy and cell fractionation, we show that in HEK 293 cells, endogenous hCtr1 is not significantly internalized in response to elevated copper. We compare the effects of extracellular copper on endogenous and over-expressed hCtr1 in HEK293 cells and show that over-expression may artificially induce relocalization of hCtr1, which is exacerbated by the presence of antibodies. Our results suggest that regulation of copper levels in mammalian cells is most likely to occur by alterations in the localization of proteins mediating exit rather than uptake.

5.3 METHODS

5.3.1 *Cell lines and expression vectors.*

Sf9 cell lines were established as previously reported (Eisses, Chi et al. 2005). Mammalian over-expressionof hCtr1 was carried out in Flp-In TREx 293 cells. Cells were purchased from Invitrogen Corporation (Carlsbad, Ca.) for use in the T-REx system, a tetracycline inducible expression system. Flp-In T-Rex -293 cells contain a single stably integrated Flp recombinase target site and also express the tetracycline repressor protein from an inducible stably integrated expression vector , pcDNA6/TR. The FLAG/N15Q mutant was cloned into a shuttle vector, pcDNA5/FRT/TO, as an AflII-XhoI fragment. The cDNA was PCR amplified to contain an amino terminal FLAG epitope tag for identification of overexpressed protein in western blot analysis as well as in immunofluorescence. The N15Q construct was co-transfected with an expression vector containing the Flp recombinase, pOG44, for site specific recombination into these engineered HEK 293 cells. Inducible expression was tested and is shown in Figure 1A.

5.3.2 *CT-hCtr1 Antibody Specificity.*

Antibody raised against the carboxyl-terminus (CT) of hCtr1, CT-hCtr1 antibody, was generated and affinity purified from rabbits by Affinity BioReagents, Golden, CO. 10 μg of antibody was incubated with 50 μg of either CT-hCtr1 peptide (SWKKAVVVDITEHCH) or an unrelated peptide from the amino terminus of the β–subunit of sheep Na, K ATPase for 48 hrs at 4°C with rocking. Adsorbed antibody was then used as the primary antibody mixture for western analysis of hCtr1 protein from HEK 293 cells. 30 μg of endoplasmic reticulum, (ER), Golgi and plasma membrane (PM) fractions from HEK 293 cells (in triplicate) were analyzed on a 12% SDS PAGE

gel and blotted to nitrocellulose. The nitrocellulose membrane was divided into thirds and probed with adsorbed antibody solutions from either hCtr1 peptide, β–subunit peptide or no peptide. Goat anti-rabbit HRP secondary antibody was used at a dilution of 1:10,000 (Pierce, IL).

5.3.3 *Cell fractionation and western analysis.*

Cells were fractionated by one of two different methods. In the first, a five-step sucrose gradient was utilized as reported previously (Gatto, McLoud et al. 2001; Eisses and Kaplan 2002; Eisses, Chi et al. 2005). In the second we utilized linear density gradients using Optiprep solution (60% (w/v) solution of iodixanol in water) from Sigma. Briefly, cells are washed twice with phosphate-buffered saline and once in homogenizing buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Hepes-NaOH, pH7.4). Cells are resuspended in 1 ml homogenization buffer and are disrupted by dounce homogenization (20 strokes) and centrifuged at 1,000 xg to remove unbroken cells and large debris. The linear gradient is formed using a gradient maker to mix a 25% (w/v) iodixanol solution with homogenization medium. The vesicle suspension is layered on top of the gradient and centrifuged at 200,000 xg for 2.5 hours. The gradient is collected in 0.5 ml fractions and analyzed by western analysis, which was typically performed using 20-30 μg of protein loaded on a 12% SDS PAGE gel. Proteins were blotted to nitrocellulose and probed with CT-hCtr1 antibody at a 1:50,000 dilution (or other antibody at specified dilution). Secondary antibodies were used at a 1:10,000 dilution.

5.3.4 *Surface biotinylation of hCtr1.*

Cells were grown until 90% confluent on T75 culture flasks and then were washed three times with phosphate –buffered saline (PBS) to remove media. Cells were

incubated at room temperature for 30 minutes with EZ-link-Sulfo-NHS-SS biotin at a concentration of 1.5 mg/ml in PBS. The cells were then washed twice with PBS to remove biotin solution and incubated in media (– serum) containing 100 μM $CuCl_2$ or in media alone for three hours at 37°C, 5% CO_2. Cells were harvested by scraping into ice-cold PBS and centrifuged at 500 xg. Cells were washed in PBS and resuspended in 500 μl of homogenization buffer. Cells were then processed for fractionation as previously described (Eisses and Kaplan 2002). ER and Golgi fractions were combined and protein concentrations for both PM and ER/G fractions were determined. Both ER/G and PM were solubilized in n-Dodecyl β-D-Maltoside (DDM). Equivalent amounts of protein from each fraction were incubated with ImmunoPure immobilized streptavidin (Pierce, Il.). Equivalent volumes were loaded onto SDS PAGE gels and analyzed by western blot analysis.

5.3.5 *si RNA and Immunofluorescence confocal microscopy.*

Cells were seeded onto glass coverslips as previously described (Eisses, Chi et al. 2005). RNAi experiments were performed as previously described (Eisses, Chi et al. 2005), and then were processed for immunofluorescence. Briefly, cells were seeded and then transfected with hCtr1-specific siRNA oligonucleotides or the LacZ gene (control) as previously reported. Cells were incubated with siRNA for 30 hrs and then fixed and stained with the antibodies described in the Figure legends.

To evaluate whether or not copper altered the location of FLAG/N15Q hCtr1 protein, induced (0.25 μg/ml tetracycline) cells were seeded on coverslips and processed one of two ways. First, cells were incubated in the presence or absence of 100 μM $CuCl_2$ for two hours and then fixed and stained for confocal microscopy as previously described

(Eisses, Chi et al. 2005). A second method of processing FLAG/N15Q cells was to incubate cells first with anti FLAG antibody and allow binding of antibody to epitope. Free and nonspecific antibody was removed from cells by washing in an acidic buffer (100 mM glycine, 20 mM magnesium acetate, 50 mM potassium chloride, pH 2.2) as has been previously reported (Petris, Smith et al. 2003). The cells, with bound antibodies, were then incubated in the presence or absence of 100 μM $CuCl_2$ for indicated times and processed for confocal microscopy. Samples were mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, Ca). Immunofluorescence microscopy was performed on a Zeiss LSM510 confocal microscope (Carl Zeiss, Gottingen, Germany).

5.4 RESULTS

5.4.1 *Expression of hCtr1.*

We generated two cell lines that over-express hCtr1 constructs, one of these expresses the C189S mutant that has been previously characterized (Eisses and Kaplan 2002; Eisses, Chi et al. 2005). This mutant was utilized instead of Wt hCtr1 because it migrates as a monomer in SDS PAGE and therefore simplifies the analysis of experiments in sf9 cells. We have previously shown that this protein is functionally equivalent to wt hCtr1 (Eisses and Kaplan 2002; Eisses, Chi et al. 2005). A second stable cell line expressing N15Q hCtr1 was generated in the mammalian Flp-In T-REx-293 cells. The construct N15Q has the asparagine residue at the single N-linked glycosylation site replaced with glutamine. The N15Q is fully active in insect cells and HEK 293 cells (Eisses and Kaplan 2002). The Flp-In T-REx-293 cell line allows tetracycline-inducible over-expression of a Flag epitope-tagged N15Q hCtr1 mutant (FLAG/N15Q) (Figure 5.1A). We show that in the absence of tetracycline, no FLAG/N15Q (FLAG/N15Q) is present. However, with the addition of 0.1 μg/ml of tetracycline a faint band recognized by the M2-Flag antibody can be detected. It can be seen that hCtr1 is induced optimally using 0.25 μg/ml tetracycline (Figure 5.1A). Increasing the concentration of tetracycline has no significant effect on the expression of FLAG/N15Q in these cells. In tetracycline concentrations equal to and greater than 0.25 μg/ml, two bands are detected. One of these bands (~25 kDa) corresponds to hCtr1 monomer without any N-linked glycosylation. The other band (~48 kDa) is likely a dimer of hCtr1. These bands are also detected by the CT-hCtr1 antibody (data not shown).

5.4.2 *Validation of Ct-hCtr1 Antibody and characterization of hCtr1 in HEK 293 cells.*

The CT-hCtr1 antibody was raised against a synthetic peptide (see Methods). We investigated the suitability of this antibody for our studies by first examining its specificity. 1 mg of affinity-purified antibody was incubated with a 5-fold excess of the immunizing peptide or a non-related peptide (a peptide from the amino terminus of the β–subunit of the Na,K ATPase) . When CT-hCtr1 antibody is adsorbed to the immunizing peptide, CT-hCtr1 antibody loses its ability to recognize hCtr1 protein in HEK 293 cell membranes bound to nitrocellulose (Figure 5.1B). Adsorbing CT-hCtr1 antibody to an unrelated peptide has no effect on the antibody's ability to recognize bands on nitrocellulose membranes. This result suggests that the CT-hCtr1 antibody recognizes the peptide used to generate the antibody and implies that the antibody is relatively specific for epitopes found in the carboxyl terminus of endogenous hCtr1, a minor membrane protein in HEK 293 cells. This is important because in Figure 5.1B there are several bands that are recognized by the antibody. There are three such bands in Figure 5.1B. These correspond to a 35 kDa, 28 kDa and a 17 kDa band. Interestingly, two of the bands are found primarily in the plasma membrane fraction and one is found predominantly in the Golgi and ER fractions. We have recently reported specific siRNA experiments to show that ~80% of the copper uptake in HEK 293 cells is mediated by hCtr1 and that the protein band that correlates with this copper transport is the 28 kDa band found in the plasma membrane fractions. This mobility agrees with the expected size for monomeric hCtr1, modified by some glycosylation. The 17 kDa band is likely a CT degradation product. The 35 kDa band is not reduced after transfection with hCtr1 specific siRNA and is likely not related to hCtr1.

In order to further characterize these protein bands we utilized a five-step sucrose cushion to fractionate cells into plasma membrane (PM), Golgi (G) and endoplasmic reticulum (ER) pools. We have previously demonstrated the integrity of these fractions utilizing enzyme activities as markers for the enrichment of each fraction in insect cells (Gatto, McLoud et al. 2001). Here we utilize antibodies to proteins that are localized in the PM and G to demonstrate that our procedure successfully fractionates HEK 293 cell membranes into PM, G, and ER fractions (Figure 5.1C).These results suggest that there may be two different proteins that are identified by our antibody, one localized inside cells with an apparent molecular mass of 35 kDa, and a second found primarily in the PM which has a molecular mass of 28 kDa. Given that the 28 kDa band found in the PM fraction has been shown to be responsible for 80% of the copper uptake in HEK 293 cells, it is probable that this 28 kDa band corresponds to hCtr1 in HEK 293 cells.

De-glycosylation experiments provide supporting evidence for this conclusion. Confirmation of the significance of the 28 kDa band as well as of the lack of relevance of the 35 kDa band in hCtr1 characterization comes from the effects of deglycosylation of these fractionated membranes. Golgi and PM fractions from HEK 293 cells were treated with PNGase F to remove any N-linked glycosylation. hCtr1 has been shown to contain a single extracellular N-linked glycosylation site, N15, which is glycosylated in sf9 cells and mammalian cells (Eisses and Kaplan 2002; Klomp, Juijn et al. 2003). As can be seen in Figure 5.2, the 28 kDa band in the PM fractions is glycosylated and exhibits an increase in mobility following treatment with PNGase F. The ~35 kDa band found in the Golgi fractions is not affected by treatment with PNGase F.

5.4.3 *Effect of copper pretreatment on cellular location in sf9 and mammalian cells.*

An hCtr1 mutant, C189S, was stably expressed in sf9 cells and the effect of high extracellular copper on hCtr1 cellular location was examined. C189S cells were incubated in the absence or presence of 100μM copper for 2 hours in the presence of 100μg/ml of cycloheximide. The cell membranes were fractionated and the fractions resolved on 12 % SDS PAGE and analyzed by western blot (Figure 5.3A). C189S protein (~26 kDa band) is primarily located in the PM membranes of both copper-treated and untreated cells. A degradation product is also seen that has an apparent mobility of ~16 kDa. It is apparent that there does not seem to be any effect of elevated copper on distribution among the ER, Golgi and Pm fractions. Furthermore, there also does not appear to be any increase in degradation that can be ascribed to the elevation in extracellular copper.

Although there is clearly no copper-dependent relocalization of hCtr1 in insect cells under our experimental conditions, previous studies have reported such effects in mammalian cells. In order to clarify this exception to our findings, we performed further studies in HEK 293 cells. We have previously reported that there did not seem to be any major relocalization effect of copper on endogenous hCtr1 in HEK 293 cells (Eisses, Chi et al. 2005). However, we could not rule out the possibility that subtle changes in Ctr1 localization were not seen as we were using only a simple sucrose cushion fractionation procedure that may show some cross-contamination between membrane pools, and only the major membrane pools are collected (see Figure 5.1C). To address this potential problem, we then fractionated HEK 293 cell membranes on linear iodixanol gradients (Figure 5.3B, collecting samples from the entire gradient. In both experimental conditions

(in the absence or presence of elevated copper) the 28 kDa band is seen in the top two fractions which correspond to the plasma membranes.

We recently showed that the siRNA-sensitive band corresponding to functional hCtr1 has an apparent mobility of 28 kDa in SDS PAGE(Eisses, Chi et al. 2005). To assess the cellular location of this siRNA-sensitive band, we investigated the knock down of hCtr1 by siRNA using confocal microscopy (Figure 5.4). Cells were seeded as described above and transfected with siRNA as previously reported (Eisses, Chi et al. 2005). Cells were either transfected with control siRNA (specific for the LacZ gene) or with hCtr1-specific siRNA. The extent of knock-down resulting from these two siRNA conditions is shown for HEK 293 cells and Flp-In TREx 293 cells expressing FlAG/N15Q (see Figure 5.4). Specific and complete knockdown of hCtr1 protein is seen at the plasma membrane in HEK 293 cells as well as Flp-In TREx 293 cells. hCtr1-specific siRNA dramatically reduces the levels of both endogenous hCtr1 (Figure 5.4 B&D) as well as over-expressed epitope-tagged, FLAG/N15Q hCtr1 in Flp-In TREx 293 cells (Figure 5.4 D&F). Residual intracellular staining by CT-hCtr1 antibody, seen in Figure 5.4 B&D is likely to be the same 35 kDa band seen in intracellular membrane fractions in westerns blots (Figure 5.1B), this staining is not evident with the anti-FLAG antibody (Figure 5.4 E&F).

5.4.4 *Surface biotinylation of hCtr1 in mammalian cells.*

The extracellular domain of hCtr1 contains a single lysine residue at position 52. We utilized this extracellular lysine to allow surface labeling of hCtr1 in HEK293 and Flp-In TREx 293 cells using a water –soluble lysine reactive biotin probe (EZ-Link-Sulfo-NHS-SS Biotin) to label hCtr1. After incubation with biotin, the cells were

incubated in media lacking serum, containing either only basal copper concentrations of copper (about 1-2μM) or 100μM $CuCl_2$. Cell membranes were obtained and fractionated and ER and Golgi fractions were combined (ER/G). Equivalent amounts of protein from the PM and ER/G fractions were solubilized in DDM and then incubated with streptavidin beads at 4°C for 6 hours. The streptavidin beads were washed and the bound protein was removed from the streptavidin beads by the addition of SDS loading buffer containing 2% βME. Equal volumes were resolved by SDS PAGE and analyzed using western blots with the CT-hCtr1 antibody. Endogenous hCtr1 in HEK 293 cells is biotinylated and is found in the PM fractions for both control and copper-treated cells (see Figure 5.5A). The apparent mobility of the band stained with the CT-hCtr1 antibody is ~100 kDa, approximately that of a trimer of endogenous hCtr1.

The results of surface biotinylation of FLAG/N15Q hCtr1 over-expressed in Flp-In TREx 293 cells are shown in Figure 5.5B. In both non-treated and copper-treated cells biotininylated hCtr1 is found in both PM and ER/G fractions. In PM fractions two bands are stained with CT-hCtr1 antibody. A band with an apparent mobility of ~100 kDa which is likely the biotinylated endogenous hCtr1 in HEK293 cells, as seen in Figure 5.5A. No significant difference in intensity for this band is seen whether cells are treated with copper or not. However, a second band is seen with an apparent mobility of ~25 kDa is also seen. This band is the expected size of FLAG/N15Q (see Figure 5.1A) running as a monomer. In copper treated cells, the almost complete loss of this 26 kDa band is seen in the PM fractions, accompanied by the appearance of a 26 kDa band in the ER/G fractions. A small amount of the 26 kDa protein is also seen in ER/G fractions from non-treated cells. The proportion of labeled ~26 kDa protein in the ER/G fraction of

non-treated cells is significantly less than the amount of protein in the ER/G fraction of copper treated cells. These results imply that internalization of hCtr1 from the plasma membrane in response to elevated copper may be seen only in the case of over-expressed epitope-tagged protein.

5.4.5 *Immunofluorescence staining of Flag N15Q hCtr1.*

The results described above from the biotinylation experiments were surprisingly similar to those presented in previous reports of copper-dependent regulation of hCtr1 and its relocalization to internal membranes (Petris, Smith et al. 2003; Guo, Smith et al. 2004). We examined the experimental conditions that might produce such effects using immunoflourescence. Cells expressing FLAG/N15Q hCtr1 were seeded onto coverslips and were either incubated with high extracellular copper (100 mM $CuCl_2$) or were incubated in media containing no added copper for 2hrs at 37°C, 5% CO_2. The cells were then fixed and permeabilized and stained for hCtr1 expression using both our CT-hCtr1 antibody and the M2-FLAG antibody. The CT-hCTr1 antibody would stain both endogenous protein and epitope-tagged protein, while the M2-FLAG antibody would only detect the over-expressed epitope-tagged protein. Intense hCtr1 plasma membrane staining is seen under basal copper conditions (Figure 5.6 A, C, and E) using both CT-hCtr1 antibody as well as the M2-Flag antibody. Following the presence of high extracellular copper, strong plasma membrane hCtr1 is detected with CT-hCtr1 antibody. However, M2-FLAG antibody staining of protein occurs not only at the plasma membrane, but also appears to have a vesicular presence in close proximity to the plasma membrane. However, the majority of hCtr1 still seems to be at the plasma membrane. Previously reported instances of rapid internalization were obtained under different

experimental conditions than we reported, namely that antibody was bound prior to copper treatment and detecttion, rather than merely being used to localize protein (Petris, Smith et al. 2003). We then tested the effect of copper on FLAG/N15Q hCtr1 protein that had antibody bound to the extracellular domain of hCtr1. FLAG/N15Q expressing-cells were seeded and incubated with M2-flag antibody at a concentration of 5 μg/ml for 10 min. The buffer was removed and cells were washed using an acidic glycine buffer as previously reported (Petris, Smith et al. 2003). Cells were washed and the incubated in media containing basal or excess copper for the indicated times (see Figure 5.7). Strikingly, under these conditions there is a dramatic increase in the extent to which elevated copper causes internalization of hCtr1. At extended times, the epitope-tagged protein is essentially cleared from the plasma membrane (See Figure 5.7), in a similar fashion to that reported previously (Petris, Smith et al. 2003), but with significantly slower rates of internalization. Thus, FLAG/N15Q cells incubated with antibody prior to copper treatment show a dramatic increase in the internalization of FLAG/N15Q hCtr1 protein compared with a similar treatment in the absence of antibody. A more modest effect is seen in cells under basal copper conditions.

5.5 DISCUSSION

We have shown that endogenous hCtr1 transports copper at the plasma membrane in HEK 293 cells and that internalization is not a necessary step in the mechanism copper transport mediated by hCtr1. These findings contradict the previous suggestions that copper transport mediated by hCtr1 utilizes an endocytic mechanism (Petris, Smith et al. 2003). The endocytosis-dependent mechanism was proposed when Petris et al reported that hCtr1 undergoes a rapid (in as little as 5- 10 minutes) and complete internalization of hCtr1 in the presence of low concentrations of copper (as little as 2 μM). This is in contrast to reports by our group and others that hCtr1 is capable of mediating linear copper uptake for at least 60 minutes (Eisses and Kaplan 2002; Lee, Pena et al. 2002). We determined that hCtr1 acts as a conventional transport protein providing a permeation pathway at the plasma membrane (Eisses, Chi et al. 2005). We did not see any significant decrease in transport function or any loss of hCtr1 protein from the plasma membrane in response to elevated extracellular copper.

In this study we showed by using a CT-hCTR1 antibody, that endogenous hCtr1 runs as a 28 kDa band in SDS PAGE. An additional band (~35 kDa) is also recognized by our antibody, but is found primarily in intracellular membranes. The 28 kDa band, but not the 35 kDa band, is sensitive to PNGase F treatment, as expected for hCtr1 and showing that hCtr1 is glycosylated through an N-linked site in HEK 293 cells as previously shown in other cellular expression systems (Eisses and Kaplan 2002; Klomp, Tops et al. 2002). Using RNAi and confocal microscopy protocols, we confirm that this 28 kDa band is located at the plasma membrane and this localization is not altered by elevations in extracellular copper levels. In contrast to results with endogenous hCtr1,

we show that over-expression of hCtr1 stimulates internalization in a copper-dependent manner. The FLAG/N15Q hCtr1 mutant is primarily localized to the plasma membrane but trace amounts of FLAG/N15Q are also found in an intracellular location. Unlike endogenous hCtr1, the over-expressed FLAG/N15Q hCtr1 protein appears to become internalized to a greater extent in the presence of high extracellular copper concentrations.

We have used two expression systems in this study to compare endogenous hCtr1 with over-expressed hCtr1. Our insect cell expression system has the advantage of producing large quantities of expressed protein, we also used a mammalian over-expression system to investigate questions about protein localization and regulation.

Our mammalian expression system utilizes site specific recombination by the Flip recombinase protein from yeast. An advantage of utilizing this particular cell line is that cDNA constructs are integrated at a specific single site in the cells genome. This allows the generation of many different mutations that can be directly compared to each other because there are no differences in genomic location of the various inserted cDNA's (i.e. transcriptional constraints due to chromatin environment) or cDNA insert copy number. A second advantage is that expression is regulated in an inducible manner by the addition of tetracycline. This allowed us to control over-expression levels. This system has allowed us to make comparisons between the lack of copper dependent regulation of endogenous hCtr1 and the putative regulation of over-expressed hCtr1 protein in a mammalian system.

Endogenous hCtr1 is localized to the plasma membrane and remains at the plasma membrane where it can mediate copper uptake even in the presence of high extracellular

copper. We show by fractionation techniques that endogenous hCtr1 (28 kDa) does not internalize or degrade when exposed to high extracellular copper concentrations (100 μM). This lack of internalization occurs in the presence of the protein synthesis inhibitor cycloheximide, discounting the possibility that new protein synthesis masks the internalization and degradation of hCtr1. We utilized linear gradient fractionation techniques to investigate the endogenous hCtr1 internalization. Similar to results reported for sucrose step gradients (Eisses, Chi et al. 2005), hCtr1 protein is localized in the plasma membrane in both copper depleted conditions as well as copper replete conditions. Linear gradients give a more complete presentation of a protein's location in the cell. In our case, protein samples throughout the gradient were examined by SDSPAGE and western blot analysis to monitor internalization of endogenous hCtr1. No significant internalization of endogenous hCtr1 was detected in the presence or absence of high extracellular copper.

Previous work in our lab utilized RNAi protocols to demonstrate that functional hCtr1 migrates as a 28 kDa band in SDS PAGE gels. In the present work confirmed the localization of this 28 kDa band by combining RNAi experiments with confocal microscopy. When HEK 293 cells are transfected with control siRNA (specific for LacZ gene) and then probed with CThCTr1 antibody, plasma membrane and intracellular staining occurs. To determine which of these locations contains the 28 kDa protein, we transfected HEK 293 cells with siRNA specific for hCtr1 and then examined the knock down of hCtr1 by confocal microscopy. There was a complete loss of plasma membrane staining. It is difficult to determine if intracellular staining also decreased. We suspect that a significant portion of the intracellular staining is due to the presence of the 35 kDa

protein. We also confirmed the plasma membrane location of overexpressed FLAG/N15Q hCtr1 utilizing the siRNA strategy.

Cell surface biotinylation experiments help to explain the difference in regulation of endogenous hCtr1 as compared to over-expressed hCtr1. We find that biotin-labeled FLAG/N15Q hCtr1 protein is found predominantly at the plasma membrane but also in intracellular compartments in the presence of basal copper levels. This is in contrast to results seen with biotin labeling of endogenous protein which is confined to the plasma membrane. Furthermore, incubation of cells over-expressing FLAG/N15Q hCtr1 in media containing high copper concentrations markedly increases the internal localization. This is reminiscent of results reported for over-expressed hCtr by other groups (Petris, Smith et al. 2003). Confocal microscopy of cells over-expressing FLAG/N15Q hCtr1 confirms this result. In high copper conditions, our over-expressed protein is re-localized to intracellular compartments (Figure 5.6). It is as yet unclear why over-expressed protein behaves differently in response to copper than does endogenous hCtr1. However, we propose that over-expression (i.e. abundance of protein) in itself may be responsible for an increase in internalization from the plasma membrane. The presence of non physiologic levels of membrane proteins in cells may artificially induce cellular responses not present at lower protein levels. Recently, a similar phenomenon was reported for the CFTR protein (Varga, Jurkuvenaite et al. 2004). In this report, endogenous CFTR matures in a different way than has been reported for CFTR protein produced in over-expression systems. Endogenous CFTR is efficiently glycosylated and is very stable in Calu-3 and T84 cells. The results presented by Varga et al show that ER associated degradation plays little or no role in the degradation of wild type endogenous

CFTR (Varga, Jurkuvenaite et al. 2004). Their work suggests that endogenous CFTR expression and post translational processing as well as the stability of expressed protein is not faithfully reproduced in heterologous over-expression systems.

Even though hCtr1 over-expression may effect cellular processing or regulation of hCtr1 in HEK293 cells, we still do not see the rapid internalization at low (physiologically relevant) copper concentrations that has been reported (Petris, Smith et al. 2003). Physiological copper concentrations range from 12- 20 μM. but most copper in serum is believed to be bound to protein or small molecular weight peptides. Our findings require high non- physiological extracellular copper concentrations (100 μM $CuCl_2$), and incubations of 2 hours to see modest internalization. When we duplicate the experimental conditions of Petris et al, utilizing overexpressed protein we can induce relatively rapid internalization in the presence as well as the absence of copper. Dramatic increases in the internalization of over-expressed protein were detected when cells were labeled with antibody prior to the addition of copper (Figure 5.7), as reported by Petris et al. This kind of observation has precedence in the literature. Two groups studying the transferrin receptor have reported a rapid redistribution of the transferrin receptor and degradation that is induced by either crosslinking or binding of antibody to the extracellular domain of the transferrin receptor (Lesley and Schulte 1985; Weissman, Klausner et al. 1986; Marsh, Leopold et al. 1995). This phenomenon has also been shown for the insulin receptor (Grunfeld 1984) as well as mannose-6-phospate receptor (von Figura, Gieselmann et al. 1984). We propose that the rapid internalization of FLAG/N15Q seen after surface antibody labeling prior to copper incubations occurs by a similar mechanism and is not relevant as a mechanism for regulating endogenous levels of hCtr1 under

physiologically relevant conditions. In other words, the reported regulatory internalization of copper is dramatically increased by examining over-expressed protein and by pre-binding antibody at the cell surface. In the absence of these confounding factors we see little evidence of copper-dependent regulatory internalization of hCtr1.

5.6 ACKNOWLEDGEMENTS

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Figure 5.1A

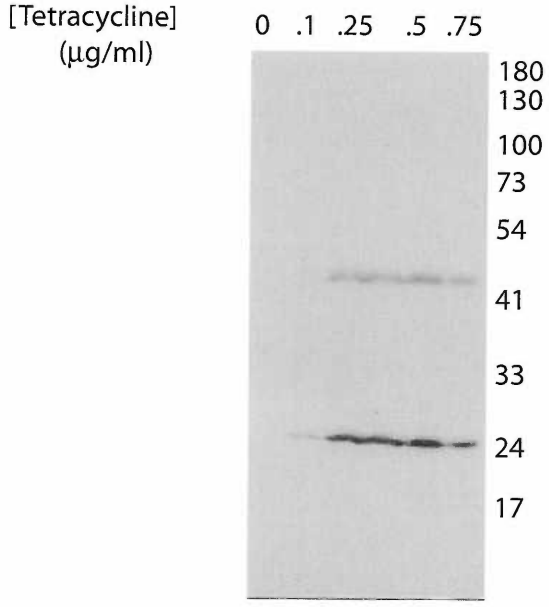


Figure 5.1B

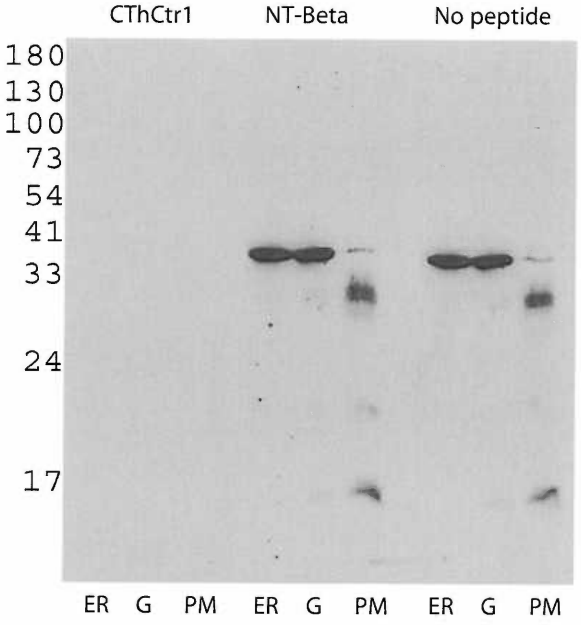

CThCtr1
NT-Beta
No peptide
180
130
100
73
54
41
33
24
17
ER G PM ER G PM ER G PM

Figure 5.1C

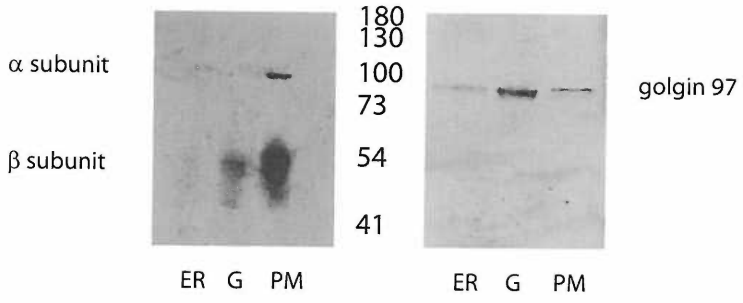


Figure 5.2 PNGase F treatment of endogenous hCtr1 protein in HEK 293 cells.

50μg of membranes from either the golgi or PM fractions of HEK 293 cells were treated with PNGase F to remove N-linked glycosylation. Protein glycosylation was monitored by SDS PAGE and western analysis using CT-hCtr1 antibody (1:25,000).

Figure 5.2

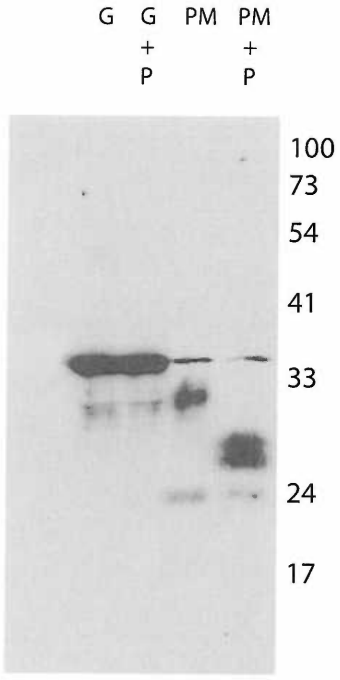


Figure 5.3 Western analysis of hCtr1 protein following copper treatment.

A) hCtr1 expression in sf9 cells.

Mutant C189S hCtr1 was stably expressed in sf9 cells. Cells were incubated with or with out 100 μM $CuCl_2$, in the presence of 100 μg/ml cycloheximide for 2 hrs at 27°C. Cells were fractionated and 20μg of each fraction was run on SDS PAGE. Blotted membranes were probed with CThCtr1 antibody.

B) Endogenous hCtr1 protein expression in HEK 293 cells.

HEK 293 cells were grown until 90 % confluent. Cells were incubated with or without excess copper in the presence of 100 μg/ml cycloheximide. Cells were fractionated on an iodixanol linear gradient. 500 μl aliquots were collected. 20 μg of each fraction was loaded on 12 % SDS PAGE gel and protein was detected using CThCtr1 antibody.

Figure 5.3A

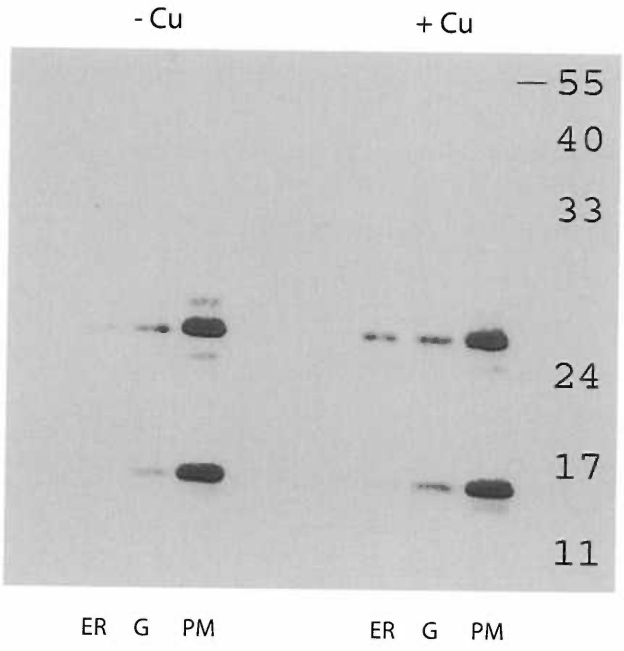


Figure 5.3B

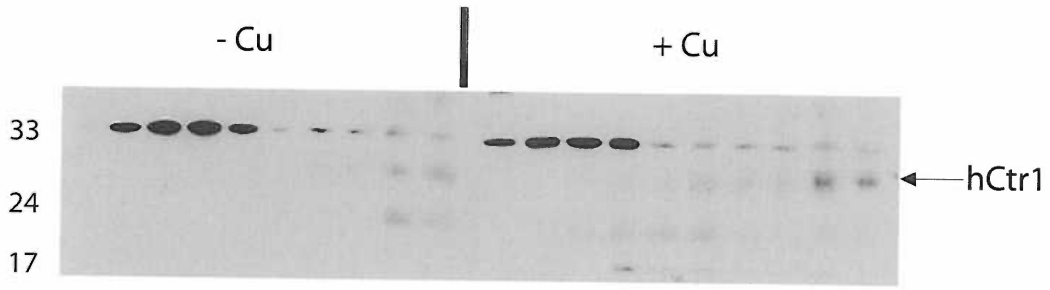


Figure 5.4 hCtr1 specific siRNA knockdown in HEK 293 and Flp-In TREx 293 cells.

siRNA specific for either the LacZ gene (control; panels A,C and E) or hCtr1 (panels B,D and F) were transfected into cells. Cells were fixed and permeabilized prior to probing with either the CThCtr1 antibody (panels A-D) or M2-FLAG antibody (panels E and F). Fluorescent secondary antibodies were used to detect either hCtr1 (Cy3 labelled goat anti rabbit, red) or M2-Flag antibody (Cy5 labelled donkey anti mouse, blue).

Figure 5.4

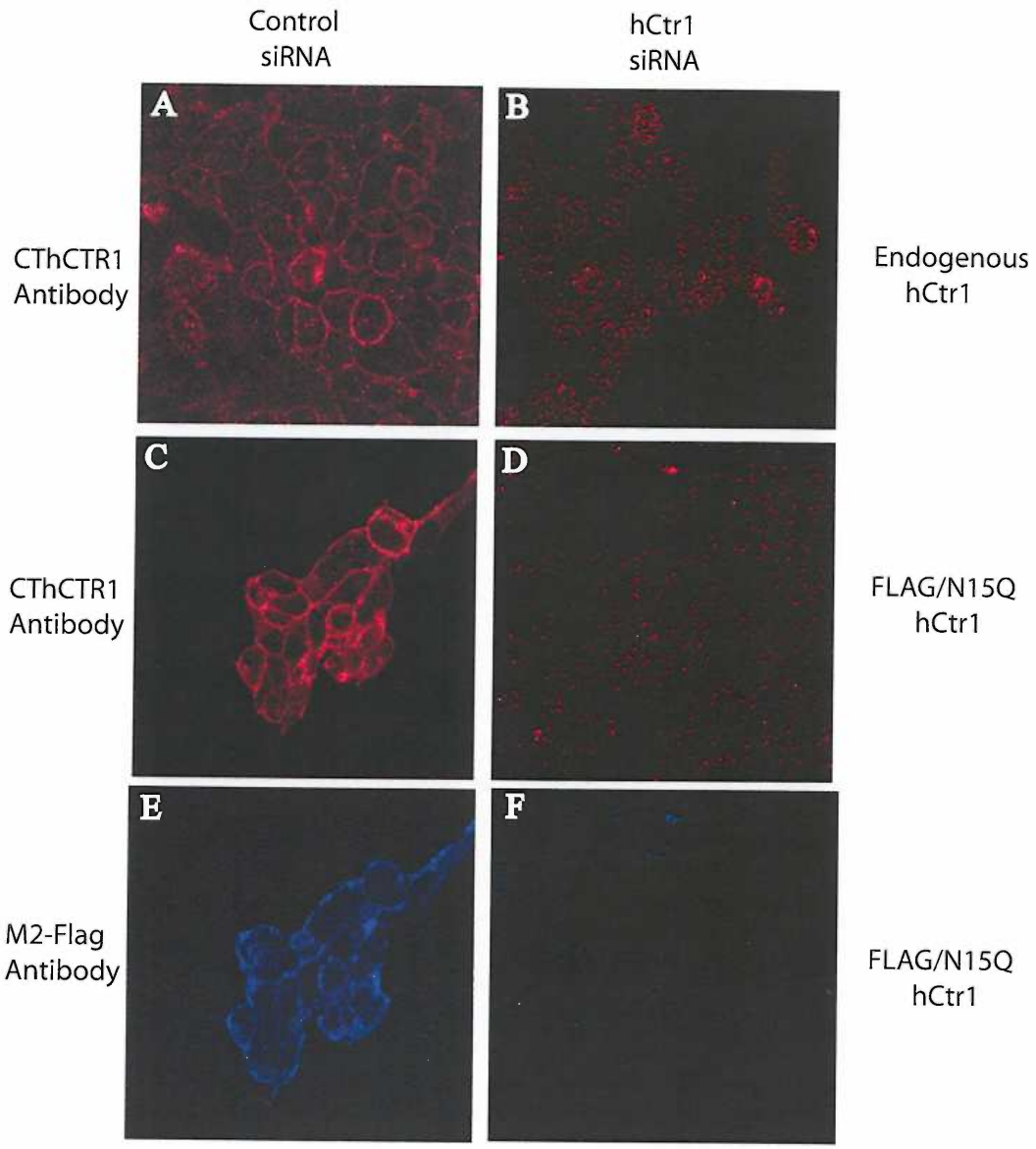

Control siRNA
hCtr1 siRNA
A
B
CThCTR1 Antibody
Endogenous hCtr1
C
D
CThCTR1 Antibody
FLAG/N15Q hCtr1
E
F
M2-Flag Antibody
FLAG/N15Q hCtr1

Figure 5.5 Surface biotinylation of hCtr1 in HEK 293 and Flp-In TREx 293 cells.

The extracellular domain of endogenous hCtr1 or FLAG/N15Q hCtr1 were biotinylated as described in the methods.

A) Biotinylation of endogenous hCtr1 in HEK 293 cells.

After incubation with the biotin reagent, cells were fractionated and equal quantities of membranes from ER/G or PM fractions were precipitated using streptavidin beads. After the pull down with streptavidin agarose, equal volumes are loaded on SDS PAGE gels and the hCtr1 protein pulled down by the beads is detected using CThCtr1 antibody.

B) Biotinylation of overexpressed epitope tagged hCtr1. A hCtr1 construct containing a Flag epitope was overexpressed in FlpIn TREx 293 cells by inducing expression in the presence of .25 mM tetracycline for 27 hours at 37°C, 5% CO_2. Biotinylation was performed as above in A.

Figure 5.5

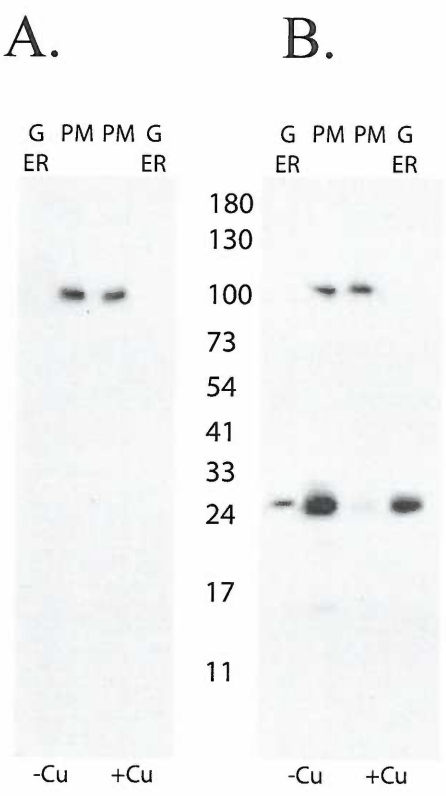


Figure 5.6 Cellular location of FLAG/N15Q hCtr1 protein in Flp-In TREx 293 cells.

The cellular location of FLAG/N15Q was investigated in basal and excess extracellular copper conditions. After copper pretreatment, hCtr1 protein was labeled using either CThCtr1 (1:1000) or M2-FLAG (1:500) antibodies. hCtr1 protein was detected using fluorescent secondary antibodies that bind to CThCtr1 (panels A and B; Cy3 goat anti rabbit, red) or M2-FLAG (panels C and D; FITC goat anti mouse, green) antibodies. Panels E&F are a merge of either A and C (E) or B and D (F).

Figure 5.6

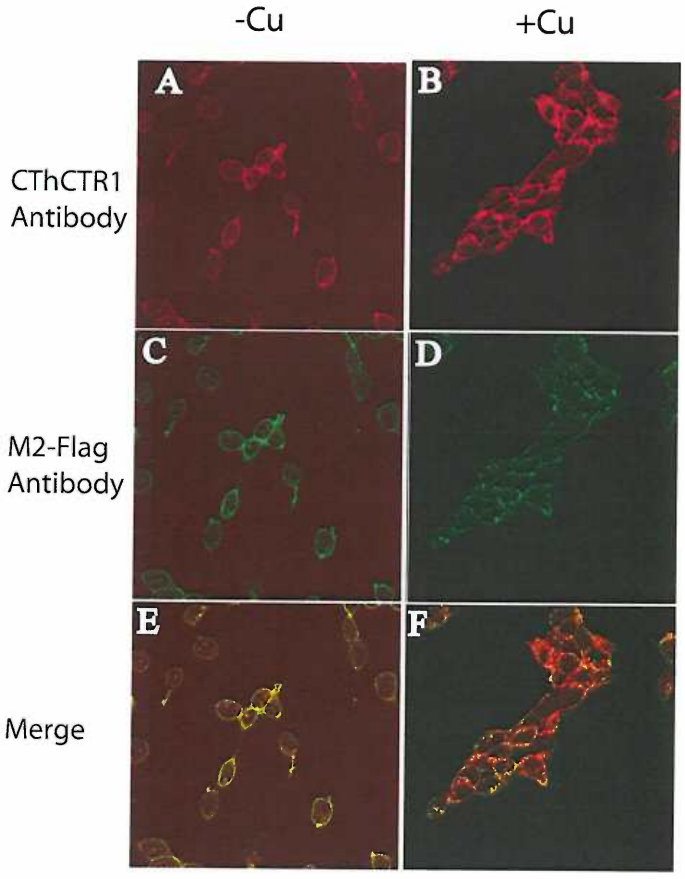
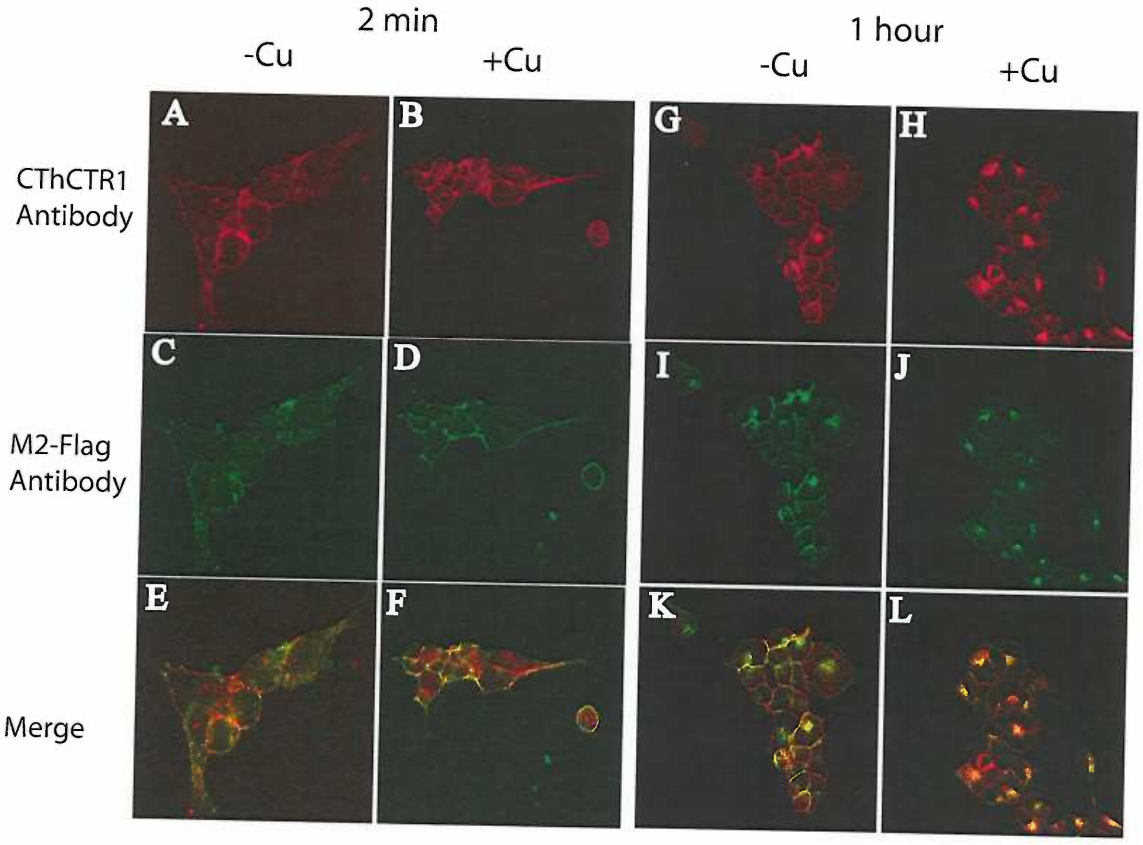


Figure 5.7 Localization of FLAG/N15Q labeled with CT-hCTr1 and M2-FLAG antibodies in basal and excess copper conditions.

Cells expressing FLAG/N15Q in Flp-In TREx 293 cells were labeled with M2-FLAG antibodies, washed with acidic buffer to remove unbound antibodies and the incubated in the presence or absence of 100 μM $CuCl_2$ for designated time periods. Cells were fixed and permeabilized. FLAG/N15Q hCtr1 protein was detected using fluorescent secondary antibodies that bind to either CT-hCTr1 (Cy3 goat anti rabbit, red) or M2-FLAG (FITC goat anti mouse, green) antibodies. A merge of results from the detection of hCtr1 protein by the two antibodies is shown.

Figure 5.7


2 min
1 hour
-Cu
+Cu
-Cu
+Cu
CThCTR1 Antibody
M2-Flag Antibody
Merge
A
B
G
H
C
D
I
J
E
F
K
L

Chapter 6

Summary and Conclusions

Copper is an essential element in humans and it is required for cellular respiration, iron homeostasis, pigment formation, neurotransmitter biosynthesis, peptide biogenesis, connective tissue production and antioxidant defense in humans. This essential character of copper is demonstrated by the various human diseases that are associated with copper as a cofactor. This association with these human diseases has raised the awareness of metals, specifically copper, and the importance of how copper contributes to the proper or improper function of copper containing enzymes. Initial studies led to the identification of novel molecules and complex pathways involved in copper distribution and led to the interest in understanding the biochemical and physiologic role of copper in organisms. The focus of this dissertation is a characterization of the uptake of copper mediated by hCtr1.

6.1 *Structure-function studies of hCtr1*

6.1.1 *Topology of hCtr1*

The amino acid sequence of hCtr1 when examined by hydropathy analysis gives three clear transmembrane segments, which thus predicts either of the two topologies. Only extracellular sites of plasma membrane proteins become glycosylated at N-X-S, N-X-T consensus sequences in the lumen of the endoplasmic reticulum. hCtr1 contains two such sites and the sequences are separated by a single transmembrane domain. In order to distinguish between the two putative membrane orientations of hCtr1, we constructed substitution mutants at N15 and N112. Our data using deglycosylation enzymes show that the correct orientation for hCtr1 has the amino terminus extracellular. To further characterize this suggested topology, we used hCtr1 molecules that contained the FLAG epitope fused to either the amino- or carboxy-terminus of hCtr1. By probing cells that

were expressing epitope-tagged hCtr1 with a Flag antibody before and after cell disruption, we could ascertain which end of the molecule was extracellular and which was intracellular. Our antibody labeling of epitope tagged hCtr1 confirms a topology where the amino terminus is extracellular and the carboxyl terminus is intracellular.

We shown that hCtr1 is susceptible to trypsin treatment. Trypsin treatment of hCtr1 in the absence of Cu produces a stable carboxyl terminal 10 kDa fragment. The predicted mass of a tryptic fragment cleaved after either K87 or R95 is 11.6 or 10.7 kDa respectively. The observed size of the C-terminal fragment clearly agrees most closely with cleavage at these intracellular sites. In the presence of 10 μM $CuCl_2$, the full-length hCtr1 molecule is protected from digestion suggesting that a conformational change in the cytoplasmic loop between transmembrane segments 1 and 2 has occurred following substrate binding.

3.5.1 *The importance of the amino-terminus of hCtr1*

Others have suggested that the amino-terminus plays an important role in copper uptake, specifically involved in the "sensing" of extra-cellular copper. We have generated several amino-terminal truncations of hCtr1 (Chapter 3). Loss of extreme NT residues residues (1-33) does not seem to have affected copper transport. Importantly, the loss of the entire extra-cellular domain (residues M1 to M69) results in a complete loss of copper uptake. This suggests that the residues that reside between the second methionine repeat (M40-M45) and the first trans-membrane domain (beginning approximately at G67) are crucial for copper transport. Several possible reasons for the importance of this region can be presented. It has been claimed, via yeast two hybrid assays, that the amino terminus is able to interact with itself and therefore may be involved in functional

oligomerization of monomers (Klomp, Juijn et al. 2003). The amino acids in this region of hCtr1 (residues F47-A66) are highly hydrophobic in many of the Ctr1 proteins that are available for protein alignment (11 hydrophobic residues out of 20in hCtr1, Figure 1A). These residues of hCtr1 may be associated with the membrane and provide important structural interactions with transmembrane segment to stabilize the functional hCtr1 complex. The associations among the amino-terminal segments at the outside surface of the plasma membrane may bring together important copper-coordinating residues that serve to steer or accumulate copper in the vicinity of a pore formed between the monomeric units.

6.1.2 *The importance of the third transmembrane segment and carboxyl terminus in hCtr1*

In the other closely related yeast proteins, Ctr1 and Ctr3 (Pena, Puig et al. 2000), there are numerous cysteine residues that may play a role in Cu transport. hCtr1 has only two cysteine residues and our work shows that neither individual cysteine is essential for function but may be important for proper organization of hCtr1 monomers in a larger homo complex. Because hCtr1 has only 190 amino acids and contains only three membrane segments, it has been suggested that the functional form of hCtr1 is probably a multimer. Pena *et al.* have shown that yCtr3 (Pena, Puig et al. 2000) and more recently hCtr1 does indeed form multimers (Lee, Pena et al. 2002). Our cysteine-substitution mutants do provide some evidence that the oligomerization of hCtr1 may be mediated through interactions at its carboxy-terminal end and these interactions may play a role in the formation and stabilization of functional hCtr1 complexes. In support of this, the C189S mutant shows only monomeric species when run on SDS gels (see Chapter 2). If

functional hCtr1, by necessity, forms multimers, other interactions must occur to facilitate their formation in the native state.

Interestingly, the double mutant C161S/C189S is significantly reduced in its ability to transport copper. This is surprising because neither of the single mutations caused a significant reduction in the ability of hCtr1 to transport copper into the cell. A possible explanation of this result is that mutations in the carboxyl terminus ie. C189 affect multimerization of hCtr1 mildly, however secondary mutations in the third trans-membrane domain exacerbate this effect. Others have shown that mutations in the third transmembrane domain disrupt multimer formation (Aller, Eng et al. 2004). Therefore if both the carboxyl terminus and the third transmembrane domain are important for helix packing and for structural integrity allowing efficient mutimerization of Ctr1 proteins, multiple mutations in these regions may significantly disrupt the ability to provide a transport pathway for copper.

The importance of the carboxyl terminal tail is demonstrated by Carboxyl terminal truncations. We show that carboxyl terminal truncations of hCtr1 are unable to transport copper at wt hCtr1 levels. The Km values are similar to wt hCtr1 but the rates of transport for the two mutants were less than half the rate of full length hCtr1. This suggests that the carboxyl terminal portion of the protein may play a role structurally, stabilizing a functional multimer complex. Additionally, we can not rule out that the carboxyl terminus of hCtr1 plays a role in the transfer of copper to intra-cellular copper homeostatic components, and that this transfer is a requirement for efficient transport.

6.1.3 *Important residues in the transmembrane segments that impact copper transport*

There are only a few residues of hCtr1 that are conserved in all Ctr1 proteins. We have mutated several of these residues in the transmembrane domains of hCtr1. Our studies have focused on potential copper coordinating residues as well as charged residues found in the transmembrane segments of hCtr1. Puig et al have suggested that the two methionine residues (conserved in all Ctr proteins, M150, M154) in the second trans-membrane domain are essential for copper transport (Puig, Lee et al. 2002). In our experiments, M150 and M154 are important for the transport of copper, however they are not essential. We see radio-isotopic uptake of copper at 28% of wt hCtr1 when these residues are replaced with isoleucines. While this underscores the importance of these two amino acids, the M150I/M154I double mutant hCtr1 is capable of transporting copper into the cell. Mutations of the two other methionine residues (M69I and M81I) show small reductions in the transport of copper into the cell (M81I more than M69I); however, they are not reduced to the same extent as the double M150I/M154I mutant protein.

It is known that tyrosine residues are important structurally as hydrogen bond partners in proteins and can also participate in metal ion coordination (Arnesano, Banci et al. 2003{Whittaker, 2005 #583).We observed only small reductions in rate of copper transport when Y83 or Y147 were replaced by alanine residues (see chapter 3). The mutation of Y156 to alanine, however, had a dramatic effect (25% of Wt protein) on the transport of copper mediated by hCtr1. Y156A only transported at a rate of about 25% of wt hCtr1. This residue is probably in the short loop between trans-membrane domain 2 and 3, and is very close to methionine 154 at the extra-cellular boundary of the putative

transport pore. Tyrosine 156 may play a structural role helping to stabilize methionine 154 for proper coordination of copper ions as they pass through the translocation pore.

There are only three charged residues in the trans-membrane domains of hCtr1. These residues are located in the first trans-membrane domain (E68, E84) and the second trans-membrane domain (H139). We see modest increases in the transport rates for the two glutamic acid residues when they are replaced with uncharged residues. This is surprising because rarely are charged residues found in transmembrane segments that have little effect on protein function when mutated. The modest increases in transport rates may signify a role in copper movement through the membrane. The change in transport for these mutants is potentially interesting as these charged residues may be important for coordinating the copper ion as it passes through the membrane or for proper placement of the copper ion at the extra-cellular domain (E68) and intra-cellular domain (E84) respectively. Given the negative charges of the side chains of these residues, it is possible that copper ions are retarded at these locations in the wild type protein as the ion passes through the membrane.

The most dramatic effect is seen with replacements at position H139. If H139 is mutated to an arginine dramatic changes both in rate of transport and the affinity for copper result suggesting the importance of this residue for copper transport. The Vmax for transport increases over 400% with the placement of a positive charge at this position. This is surprising as the histidine is not strictly conserved between yeast and mammalian Ctr1 proteins (mammalian Ctr1 proteins have a histidine at this position, yeast Ctr proteins do not). However, there is an arginine four amino acids away (~one turn of an α helix) from this histidine in both yCtr1 and yCtr3. One possibility is that this histidine is

placed into the pore formed by the association of perhaps three monomers. Replacement with arginine residues places three fixed positive charges in the pore which may result in charge repulsions widening the pore at a critical site, lessening interactions with the permeating copper ion and increasing the maximal transport rate.

Bringing together the results from this dissertation, we can begin to develop a map of residues that are important for hCtr1 function (Chapter 3, Figures 3.7 and 3.8). We present results that identify a number of amino acids that are important for the function of hCtr1 as a copper transporter. This dissertation provides evidence of the importance of the amino terminus, carboxyl terminus and selected residues in the trans-membrane domains.

6.2 *Localization and regulation of hCtr1*

We have shown that endogenous hCtr1 transports copper at the plasma membrane in HEK 293 cells and that internalization is not a necessary step in the mechanism of copper transport mediated by hCtr1. The absence of a Cu-dependent decrease in the rate of Cu uptake and no loss of hCtr1 from the plasma membrane of insect cells confirm that internalization of Cu-bound transporter is not an essential step for Cu uptake mediated by hCtr1. It seems likely that hCtr1 mediates Cu uptake in a more conventional manner. We suggest that hCtr1 provides a Cu permeation pathway through the plasma membrane. It seems likely that a stable oligomer of hCtr1 monomers forms in the cell membrane and this complex mediates the uptake of Cu into the cell. It has been suggested that these oligomers are probably trimeric, although other structures, tetramers or trimers of dimers have not been ruled out. We suggest that the important methionine-rich domains in the extra-cellular amino-terminus (Eisses and Kaplan 2002; Puig, Lee et al. 2002; Klomp,

Juijn et al. 2003) provide a Cu-coordinating center that localizes Cu to the mouth of the trans-membrane transport pathway. hCtr1 remains at the plasma membrane and mediates this metal transport pathway.

Our results provide strong evidence that a previously proposed endocytic mechanism of transport for hCtr1 is not correct. The endocytosis-dependent mechanism was proposed when Petris et al reported that over-expressed epitope tagged hCtr1 undergoes a rapid (in as little as 5- 10 minutes) and complete internalization of hCtr1 in the presence of low concentrations of copper (as little as 2 μM). Although both labeling and confocal microscopic evidence was presented for these epitope-tagged constructs, it should be emphasized that no functional corollary was demonstrated. Studies in other cells (HeLa and Caco2 cells) did not observe such Cu-dependent relocalization of endogenous levels of hCTR1 (Klomp, Tops et al. 2002), and these authors concluded that these putative regulatory phenomena may be cell-specific.

We have used two expression systems in this study to compare endogenous hCtr1 with over-expressed hCtr1 to investigate this discrepancy between our work and others. We have utilized RNAi protocols to demonstrate that functional hCtr1 migrates as a 28 kDa band in SDS PAGE gels and have confirmed the localization of this 28 kDa band in HEK 293 cells by combining these RNAi experiments with confocal microscopy (Chapter 5, Figure 5.4).

Cell surface biotinylation experiments help to explain the difference in regulation of endogenous hCtr1 as compared to over-expressed hCtr1. We find that biotin-labeled FLAG/N15Q hCtr1 protein is found predominantly at the plasma membrane but also in intracellular compartments in the presence of basal copper levels. This is in contrast to

results seen with biotin labeling of endogenous protein which is confined to the plasma membrane. Furthermore, incubation of cells over-expressing FLAG/N15Q hCtr1 in media containing high copper concentrations markedly increases the internal localization. We propose that over-expression (i.e. abundance of protein) in itself may be responsible for an increase in internalization from the plasma membrane. The presence of non physiologic levels of membrane proteins in cells may artificially induce cellular responses not present at lower protein levels. Recently, a similar phenomenon was reported for the CFTR protein (Varga, Jurkuvenaite et al. 2004).

In this dissertation, we supply functional (Cu uptake measurements), biochemical (cell fractionation, cell surface biotinylation), RNA knockdown and confocal microscopic data that together suggest that endogenous levels of hCTR1 are stably expressed in the plasma membrane and remain functional in the face of elevated extra-cellular Cu.

We suggest that Cu uptake is mediated by hCTR1 by providing a transport pathway across the plasma membrane and that the regulation of cellular Cu content in the face of elevated extra-cellular Cu levels more likely occurs via regulation of the Cu exit pathways such as is known to occur in the re-localization of ATP7A, the Menkes disease protein from intracellular locations to the plasma membrane in response to elevated Cu levels.

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