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Model Peptides Provide New Insights into the Role of Histidine Residues as Potential Ligands in Human Cellular Copper Acquisition via Ctr1

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Abstract

Cellular acquisition of copper in eukaryotes is primarily accomplished through the Ctr family of copper transport proteins. In both humans and yeast, methionine-rich “Mets” motifs in the amino-terminal extracellular domain of Ctr1 are thought to be responsible for recruitment of copper at the cell surface. Unlike yeast, mammalian Ctr1 also contains extracellular histidine-rich motifs, although a role for these regions in copper uptake has not been explored in detail. Herein, synthetic model peptides containing the first 14 residues of the extracellular domain of human Ctr1 (MDHSHHMGMSYMDS) have been prepared and evaluated for their apparent binding affinity to both Cu(I) and Cu(II). These studies reveal a high affinity Cu(II) binding site (logK = 11.0 ± 0.3 at pH 7.4) at the amino-terminus of the peptide as well as a high affinity Cu(I) site (logK = 10.2 ± 0.2 at pH 7.4) that utilizes adjacent HH residues along with an additional His or Met ligand. These model studies suggest that the histidine domains may play a direct role in copper acquisition from serum copper-binding proteins and in facilitating the reduction of Cu(II) to the active Ctr1 substrate, Cu(I). We tested this hypothesis by expressing a Ctr1 mutant lacking only extracellular histidine residues in Ctr1-knockout mouse embryonic fibroblasts. Results from live cell studies support the hypothesis that extracellular amino-terminal His residues directly participate in the copper transport function of Ctr1.

Introduction

Copper is an important metal cofactor required for several cellular metabolic processes, including oxidative phosphorylation, antioxidant activity, connective tissue formation, pigmentation, iron metabolism, and neurotransmitter synthesis. In humans, enzymes and proteins that require copper include copper-zinc superoxide dismutase, cytochrome C oxidase, lysyl oxidase, ceruloplasmin, dopamine β-hydroxylase, tyrosinase, peptidylglycine monooxygenase, clotting factors V and VIII, angiogenin, hephaestin, and others.[1] Copper is useful in these enzymes because it can effect electron transfer reactions due to its two primary biologically accessible oxidation states, Cu(I) and Cu(II). Controlled redox cycling between these two oxidation states imparts function to the enzymes that require copper as a cofactor, however uncontrolled redox cycling due to systemic overload of copper or local

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Supporting Information Available. Immunoblots showing that Ctr1 and Ctr1ntHA are both N-glycosylated (Figure S1) and that the bands between 17–37 kDa correspond to monomeric Ctr1 proteins (Figure S2). This information is available free of charge via the Internet at http://pubs.acs.org/

dysfunction in the proteins that handle copper is known to cause debilitating human diseases. Aberrant copper is also toxic because it binds promiscuously to proteins and can occupy binding sites meant for other metals.[2,3] These aspects define a paradoxical state of copper where it is both essential to biological processes, but also potentially toxic.

The essential yet toxic nature of copper requires that it be tightly regulated in biology both intra- and extracellularly. Over the past few decades, intense investigation on the proteins that govern copper homeostasis has lead to critical progress in our understanding of the mechanisms involved in copper uptake, transport and efflux from eukaryotic cells.[1,4–9] The proteins involved in copper homeostasis are highly conserved in their structure, function and mechanisms of action across all species investigated, including plants, microbes, and mammals. It is clear from these studies that Nature has evolved to handle copper in a carefully controlled manner.[10–13]

Copper is acquired from the extracellular environment via plasma membrane copper transport proteins. Studies in the baker's yeast *S. cerevisiae* first led to identification of the copper transport proteins Ctr1 and Ctr3, founding members of a family of copper transport proteins (Ctr) that are widely conserved in yeast, fungi, plants, and mammals.[14–16] Genetic studies in yeast, Drosophila and mouse confirm that Ctr1 is required for copper acquisition.[4] Yeast have two copper transporters responsible for extracellular copper acquisition, Ctr1 and Ctr3, and one vacuolar copper transporter, Ctr2, responsible for mobilization of intracellular copper stores.[17,18] Although the human and mouse genome encode two putative copper transporters, denoted Ctr1 and Ctr2, only Ctr1 has been demonstrated to be involved in cellular copper acquisition.

Human Ctr1 is a 190 amino acid protein that localizes to the plasma membrane and contains three transmembrane domains, an extracellular amino-terminal domain and an intracellular carboxyl-terminal domain (Figure 1). A three-dimensional (3-D) structure of Ctr1 has recently confirmed that the copper transporter forms a homotrimer to create a cone-shaped pore in the plasma membrane.[19,20] The 3-D image shows that the pore opening is bordered by the second transmembrane domain (TMD2) of each monomer. TMD2 contains an essential MX_3M domain: mutagenesis of either essential methionine in TMD2 renders the protein inactive for copper transport.[21–23] The 3-D images reveal little about the structure of the amino and carboxyl-terminal domains, indicating the need for higher resolution structural information and genotype-phenotype analysis.

It is well established that Ctr1 proteins in all species examined selectively transport Cu(I) and therefore Ctr1-dependent copper uptake likely requires a cell surface reductase. In *S. cerevisiae* Cu(II) is reduced at the plasma membrane by the iron and copper reductases Fre1 and Fre2.[24,25] Although there have been suggestions that specific proteins may serve this role in mammals,[26] conclusive evidence for the identity of a mammalian cell surface copper reductase is still lacking.

Little is known about the mechanisms by which Ctr1 acquires copper from the extracellular environment and passes the metal ion across the plasma membrane to cytoplasmic copper chaperone proteins.[5] The extracellular domains of both human and yeast Ctr1 proteins contain multiple closely spaced methionine residues arranged in patterns of MXM and MX_2M, where X is one of several amino acids. These "Mets motifs" are capable of binding Cu(I) with micromolar affinity and may be responsible, in part, for recruiting Cu(I) to the Ctr1 pore.[27,28] In yeast cells Cu(II) is predicted to be first reduced by Fre1/2 at which point Ctr1 Mets motifs may bind Cu(I) and transport it through the plasma membrane. While the methionine-rich motifs of yeast Ctr1 are not absolutely required for function, they are important for cellular copper acquisition under conditions where bioavailability of copper is

limited. There is one conserved Met residue in the extracellular N-terminal domain, Met 127 in yeast, and Met 45 (or one proximal met) in human, that is required for copper transport. Apart from this one critical Met residue, the ability of Ctr1 to acquire copper in the presence of extracellular chelators roughly correlates with the number of Mets motifs present in its amino-terminus.[21] Presumably, the extracellular Mets motifs in Ctr1 are responsible for accumulation of copper at the plasma membrane, and more specifically at the Ctr1 pore opening. Especially when bioavalable copper is limited, these Mets motifs may act by functionally increasing local concentrations of copper at the pore opening, enabling passive metal transport down a concentration gradient from high [Cu] directly outside the pore, to low [Cu] in the cytoplasm.

Unlike that of yeast, human Ctr1 has histidine-rich domains that are conserved throughout several mammalian species. Early studies of Ctr1 expressed in yeast ctr1Δ cells or over-expressed in insect and HEK cell models found no role for the histidines of Ctr1 in copper uptake.[21,23] However, studies of human Ctr1 in yeast may not reveal a role for extracellular His in copper acquisition since His metal binding is pH dependent and yeast acidify their environment below pH 6 during growth. It is also possible that studies of Ctr1 over-expressed in mammalian and insect cells would not reveal a phenotype for histidine mutants due to endogenous levels of Ctr1 expression. There are compelling reasons to test whether His-rich regions in Ctr1 may be important for copper acquisition in humans. Recent evidence suggests that truncation of ~30 amino acid residues from the Ctr1 amino-terminus results in decreased Cu transport in cells either expressing endogenous levels of Ctr1 or devoid of Ctr1.[29,30] Such a truncation eliminates most of the His residues. Furthermore, the Ctr1 amino-terminus of several mammalian species, including human, contains a histidine in the third position to give an H_2N-XXH site that is known as an ATCUN motif, or “amino terminal Cu and Ni binder”.[31] This peptide arrangement creates a high affinity Cu(II) binding site that is found in the blood copper carrier, human serum albumin (HSA),[32] as well as other biologically important proteins and peptides that have been proposed to bind copper in vivo. [31,33–36]

The amino terminal extracellular domain of human Ctr1 also harbors a bis-His (HH) site that is known to bind and stabilize Cu(I) in prion and Aβ model peptides.[37–40] This bis-His cluster is bordered by a Mets motif of the form $MXMX_2M$, which alone is sufficient for Cu(I) binding,[21,27] but could also participate in a mixed binding site with nearby His residues. There are two additional His clusters and one other Mets motif within this extracellular domain. The presence of the albumin-like H_2N-XXH sequence and other His residues suggest that in addition to Cu(I) interaction through Mets motifs, human Ctr1 may be capable of interacting with both Cu(I) and Cu(II) via His residues. In this study we have characterized the Cu(I) and Cu(II) binding properties of model peptides based on the first 14 amino acids of human Ctr1. Based on predictions from model peptides, we tested the functional contribution of His residues in the context of full-length human Ctr1 knockout mouse embryonic fibroblasts. The results suggest a revised picture of mammalian copper acquisition that includes both Cu(II) and Cu(I) interactions with histidine-containing regions within the Ctr1 amino-terminal extracellular domain.

Experimental Section

Peptide Synthesis and Purification—Model peptides based on the first 14 amino-terminal amino acids of human Ctr1 were synthesized on a Protein Technologies PS3 automated peptide synthesizer on Fmoc-PAL-PEG-PS resin (Applied Biosystems) in 0.1 and 0.05 mmol scales. Couplings of standard Fmoc (9-fluorenylmethoxy-carbonyl)-protected amino acids (Chem-Impex, Novabiochem) were achieved with HBTU (O-benzotriazole-N,N,N′,N′-tetramethyl-uronium hexafluorophosphate; Novabiochem) in the presence of N-

methylmorpholine (NMM) in N,N′-dimethylformamide (DMF) for 20 min cycles. Fmoc deprotection was achieved with 20% piperidine in DMF. The amino-termini of peptides were either acetylated with acetic anhydride and NMM or were allowed to remain as a free amine. Side chain deprotection and peptide cleavage from the resin were achieved by treating the resin-bound peptides with a 5–7 mL cocktail of 95% trifluoroacetic acid (TFA), 2.5% ethane dithiol (EDT), and 2.5% triisopropylsilane (TIS) for 2–4 h under N_2. An additional 75–150 μL of EDT and 65–130 μL of bromotrimethylsilane (TMSBr) were added during the final 30 min to minimize methionine oxidation. After evaporation of TFA to a volume of 1–2 mL under N_2, the peptides were washed three times with diethyl ether, air-dried, and purified by semipreparative reverse-phase HPLC on a YMC C18 column with a linear 40-min gradient from 7 to 93% acetonitrile in water with 0.1% TFA. The purity was validated to be >95% by analytical HPLC, and the mass of each peptide was confirmed by ESI-MS. Ctr1-14 calcd for 1663.6: Found ($M + H^+$) 1664.7. Ctr1-14(HA) calcd for 1721.7: Found ($M + H^+$) 1722.8. Ctr1-14(H3A), Ctr1-14(H5A), Ctr1-14(H6A) calcd for 1853.8: Found ($M + H^+$) 1854.7. Ctr1-14(H56A) calcd for 1787.7: Found ($M + H^+$) 1788.9. Ctr1-14(H56AMNle) calcd for 1715.9: Found 1717.4. Ctr1-14(MNle) calcd for 1848.0: Found ($M + H^+$) 1849.1. Ac-Ctr1-14 calcd for 1705.6: Found ($M + H^+$) 1706.7. Ac-Ctr1-14(H56A) calcd for 1829.8: Found ($M + H^+$) 1830.8.

Preparation of Stock Solutions—Stock solutions of peptides were prepared by dissolving lyophilized peptide in 1–1.5 mL of Nanopure water. Concentration was determined using the Edelhoch method.[41,42] In short, 5–15 μL aliquots of peptide were diluted into 1mL total volume of 6 M guanidinium hydrochloride (GdnHCl) to get an absorbance at 280 nm between 0.1 and 1 absorbance unit. Absorbance of aromatic amino acids, in this case only tyrosine, was measured at 276, 278, 280 and 282 nm. Known extinction coefficients of tyrosine at these wavelengths were used to determine total concentration of peptide. Stock peptide solutions were periodically checked by HPLC and ESI-MS to verify that they remained stable to oxidation or degradation in aqueous solution. Copper(II) solutions were prepared in Nanopure water from $Cu(ClO_4)_2 \cdot 6H_2O$ (Strem Chemicals), and were standardized by EDTA (Aldrich) titration in ammonia solution with a murexide indicator (Fischer).[43] Copper(I) solutions were prepared from $[Cu(CH_3CN)_4]PF_6$ (Aldrich) in anhydrous acetonitrile (Aldrich) and were standardized by titrating aliquots of stock solution into excess of the chromophoric ligand anion, bicinchoninate (BCA), in doubly deionized water that was boiled and degassed with argon. Concentration was determined from absorption at 562 nm due to the $Cu(I)(BCA)_2$ complex ($\varepsilon = 7900\ M^{-1}\ cm^{-1}$).[44] Solutions of Cu(I) were stored under argon and re-standardized each day to ensure that Cu(I) had not oxidized.

Mass Spectrometry—Electrospray ionization mass spectrometry (ESI-MS) was performed with an electrospray quadrupole ion trap mass spectrometer (1100 Series LC/MSD Trap, Agilent, Palo Alto, CA) with a conversion dynode detector (Daly). Samples were infused with a Harvard Apparatus (Holliston, MA) syringe pump at 33 μL/min. Ionization was achieved in the positive ion mode by application of +5 kV at the entrance to the capillary; the pressure of the nebulizer gas was 20 psi. The drying gas was heated to 325 °C at a flow of 7 L/min. Full-scan mass spectra were recorded in the mass/charge (*m/z*) range of 200–2000.

UV-Visible Spectroscopy—Absorption spectra were recorded in 1-cm quartz cuvettes on either a Cary 50 UV-vis spectrophotometer or an SI Photonics (Tucson, AZ) model 420 fiber optic CCD array UV-vis spectrophotometer.

Determination of peptide-copper binding constants—Association constants in these experiments are the apparent association constants of copper ion with peptide in 50 mM HEPES buffer at pH 7.4 assuming 1:1 binding of peptide (P) and copper. The association constants for the P–Cu complexes were determined by fitting titration spectra to an appropriate equilibrium model using Specfit[45,46] software.

Cu(II) binding constants—In the case of Cu(II) binding to Ctr1 model peptides, the assumption of 1:1 P:Cu complex formation is validated by a linear relationship between absorbance maxima between 400 and 700 nm due to peptide-copper complex (PCu(II)) and ratio of P:Cu(II) up to 1:1 in 50 mM HEPES buffer at pH 7.4. More than one equivalent of Cu(II) to P in solution results in precipitation of a light blue solid (copper hydroxide) indicating that each peptide can accommodate only one Cu(II) ion in solution.

Titrations to determine peptide copper affinity were performed with nitrilotriacetic acid (NTA) (K_{CuNTA} = 10.68, pH 7.4)[47] as a competitive chelator for Cu(II). Solutions of 0.5−1 mM PCu(II) were titrated with at least two equivalents of NTA using a 100 mM NTA stock solution. For peptides that had a low $K_{PCu(II)}$ ($\log K_{PCu(II)} < 10$) titrations were performed in the reverse direction with peptide being titrated into Cu(II)NTA solution under the same conditions.

When using NTA as a competitive chelator in HEPES buffer, the experiment is complicated by the fact that HEPES buffer can interact with Cu(II)[48] and can form a ternary complex with Cu(II)NTA.[32] These interactions should result in an apparent $K_{PCu(II)}$ value that appears weaker than the actual conditional association constant value at pH 7.4.[48] Here we report apparent values at pH 7.4 in the presence of HEPES and NTA that do not account for ternary complex formation or the association of HEPES and Cu(II). Titration spectra were fit to the equilibrium model shown in Table 1, based on the expressions shown in Equations 1–7.

$$P + Cu^{2+} \leftrightarrows PCu(II) \qquad \text{Equation 1}$$

$$K_{PCu(II)} = \frac{[PCu(II)]}{[Cu^{2+}][P]} \qquad \text{Equation 2}$$

$$NTA + Cu^{2+} \leftrightarrows NTACu(II) \qquad \text{Equation 3}$$

$$K_{NTACu(II)} = \frac{[NTACu(II)]}{[Cu^{2+}][NTA]} \qquad \text{Equation 4}$$

$$PCu(II) + NTA \leftrightarrows NTACu(II) + P \qquad \text{Equation 5}$$

$$K_{ex} = \frac{[P][NTACu(II)]}{[PCu(II)][NTA]} = \frac{K_{NTACu(II)}}{K^{app}_{PCu(II)}}$$ Equation 6

$$K^{app}_{PCu(II)} = \frac{K_{NTACu(II)}}{K_{ex}}$$ Equation 7

Cu(I) binding constants—To determine peptide Cu(I) association constants, the colorimetric Cu(I) chelator BCA was employed as a competitive ligand. Although Cu(I) complexes are usually spectroscopically silent, the $Cu(I)(BCA)_2$ complex is colored and has strong absorbance at 358 nm (ε = 42900 M^{-1} cm^{-1}) and 562 nm (ε = 7900 M^{-1} cm^{-1}).[49,50] Association constants ($K_{PCu(I)}$) were determined for association of Cu(I) ion and peptide at pH 7.4 in HEPES buffer with 0.5 % acetonitrile and assuming 1:1 binding of peptide to Cu(I). Although it may be possible for peptides to bind multiple Cu(I) ions, the assumption of 1:1 P:Cu(I) stoichiometry is validated by the large excess of peptide present during the course of these experiments. Peptide stock solutions were titrated into 1 mL solutions of $Cu(I)(BCA)_2$ (prepared from 50 μM $Cu(CH_3CN)_4PF_6$ and 150 μM BCA) and competition was observed as a disappearance of the $Cu(I)(BCA)_2$ absorption bands. Calculations were done using Specfit[45,46] software and an equilibrium model shown in Table 2 and based on Equations 8–12. Two different affinity constants found in the literature for formation of Cu(I)BCA and $Cu(I)(BCA)_2$ were taken into account.[49,51] Acetonitrile has low affinity for copper (log K_1=2.63, log K_2=1.39, log K_3=0.28)[52] and formation of pure acetonitile-Cu(I) species in solution is unlikely in the presence of excess BCA and excess peptide in solution, both with affinities constants more than ten orders of magnitudes higher than acetonitrile for Cu(I). The extent to which acetonitrile can participate in ternary complex formation with the peptide-Cu(I) complex is unknown and is not addressed in these experiments. For this reason, we note that the constants reported here for Cu(I) and Ctr1 model peptides are apparent values and are limited in that they may not reflect the value of purely conditional pH-dependent constants.

$$Cu^{+} + BCA \leftrightarrows Cu(BCA)$$ Equation 8

$$\beta_1 = \frac{[CuBCA]}{[Cu][BCA]} = K_1$$ Equation 9

$$Cu^{+} + 2\,BCA \leftrightarrows Cu(BCA)_2$$ Equation 10

$$\beta_2 = \frac{[Cu(BCA)_2]}{[Cu][BCA]^2} = K_1 K_2$$ Equation 11

$$Cu(BCA)_2 + P \leftrightarrows PCu + 2BCA$$ Equation 12

Generation and Growth of Cell Lines

Mouse embryonic fibroblast (MEF) cells derived from wild type or $Ctr1^{-/-}$ mice (MEF $Ctr1^{-/-}$), and MEF Ctr1 knockout cells stably transfected with pcDNA3.1(+) expressing wild type human Ctr1 cDNA under control of the cytomegalovirus promoter (MEFΔhCtr1$^{+/+}$) are described elsewhere.[53] MEF cell lines were cultured in Dulbecco's modified eagle medium (DMEM (Invitrogen catalog #11995) supplemented with up to 20% fetal bovine serum (FBS, Gibco), 100 units/mL penicillin, 100 μg/mL streptomycin, 1X nonessential amino acids (Gibco) and 55 μM 2-mercaptoethanol. To facilitate mitochondrial function under potentially sub-optimal intracellular copper concentrations, 1 mM sodium pyruvate, and 0.5 μg/L uridine were also added to culture medium.[53] Cells were split using 0.05% trypsin incubated at 37 °C for 2 min.

A pcDNA3.1(+) plasmid containing myc-tagged human Ctr1 DNA with His cluster H1 (HSHH) mutated to A (ASAA) was generated previously.[21] The Ctr1 amino-terminus contains two other histidine clusters, which were mutated to alanine using Ctr1-specific primers sets Ctr1H2A and Ctr1H3A listed in Table 3. The H2 (HHH) cluster was mutated to alanine (AAA) using primers Ctr1H2A and Ctr1H3A-F and -R and the H3 (HSH) cluster was mutated to alanine (ASA) using primers Ctr1H3A-F and -R (Table 3) in two steps using a Quikchange® Multi Site-Directed Mutagenesis Kit (Stratagene) and a standard Quikchange® PCR protocols. In each case, after mutagenesis, the 25 μL reaction was digested with 1 μL DPN1 enzyme, ethanol precipitated and taken up in 10 μL of elution buffer. This entire sample was transformed into XL10-Gold® Ultracompetant Cells (Strategene) and cells were incubated on LB plates containing ampicillin for 16 h at 37 °C. Individual colonies were inoculated into separate sterile culture tubes containing 3 mL of LB media plus ampicillin and incubated 16 h at 37 °C. Plasmid DNA was harvested with QIAprep Spin Miniprep Kit (QIAGEN). The Ctr1 insert was amplified using primers from Table 3 to remove the myc tag and add appropriate restriction sites, XhoI and EcoR1, for ligation of the insert into pcDNA 3.1 zeo (+) plasmid (Invitrogen). DNA was transformed into XL10-Gold Ultracompetant Cells and purified using Miniprep DNA kit. Miniprep DNA was then sequenced to verify proper mutation and amplification.

$Ctr1^{-/-}$ MEF were transfected by electroporation with pcDNA3.1zeo(+) vector containing a Zeocin™ (Invitrogen) antibiotic resistance gene and the Ctr1ntHA cDNA under control of the cytomegalovirus promoter. Transiently transfected cells were put under selective pressure for stable expression using 750 μg/mL Zeocin™ (Invitrogen). After selection cells were trypsinized and harvested for selection of homogeneous stable cell lines. Cells were counted and plated in a 96 well plate at a density of 0.8 cells per well at a volume of 100 μL per well containing 750 μg/mL Zeocin™. After several days approximately 30 wells contained viable stable cell lines. When cells became confluent, they were transferred in a graduated fashion to larger wells until there were enough cells of each homogeneous stable cell line to fill the wells of 6 well flat bottom plates. These cells were harvested and Ctr1ntHA expression was assessed. These mouse Ctr1 knockout MEF cell lines stably expressing human Ctr1ntHA mutant DNA (MEF Ctr1ntHA$^{+/+}$) were selected for Ctr1ntHA expression levels similar to the expression level of hCtr1 in the MEF $Ctr1^{+/+}$ stable cell line.

Protein Isolation and Western Blotting Analysis

Confluent MEF cell cultures were rinsed twice with cold 1× PBS buffer, scraped from plates and pelleted by centrifugation at 15K rpm for 30 s. Cells were resuspended and incubated on ice for 30–60 min with 50–100 μL lysis buffer (1% TritonX100, 0.1%SDS, 1mM EDTA, and 1 cOmplete™ Mini EDTA-free protease inhibitor tablet (Roche) in 10 mL 1× PBS). Protein was isolated by centrifugation at 4 °C and 15K rpm for 10 min. Supernatant was

transferred to a clean 1.5-mL tube for protein quantification by using the Bio-Rad DC protein assay.

Samples were run on pre-cast Criterion Tris-HCl sodium dodecyl sulfite polyacrylamide gradient gels (Bio-Rad) using electrophoresis (SDS-PAGE) and transferred to a nitrocellulose or PVDF membrane. Where noted, loading buffer with and without 2-mercaptoethanol was used to determine multimerization of the Ctr1ntHA mutant compared to wild type. The membranes were blocked for at least 1 hour in 5% skim milk/TBS-T and allowed to incubate overnight in primary antibody diluted in blocking solution. Primary antibodies used are as follows: Rabbit anti-human Ctr1, described elsewhere,[54] was used at 1:1000 dilution ; rabbit anti-CCS (FL-274) from Santa Cruz Biotechnology (1:200); mouse anti-CoxIV from Invitrogen (1:1000); mouse anti-Actin from BD Biosciences (1:1000); rabbit anti-Actin; rabbit anti-Tubulin. Secondary antibodies were donkey anti-rabbit and anti-mouse conjugated with horseradish peroxidase from Amersham Biosciences (1:5000).

N-Glycosidase Treatment

The comparison of the N-glycosylation status of Ctr1 and Ctr1ntHA mutant protein was determined by treatment of total cell protein extract with N-Glycosidase F, also known as PNGaseF (New England Biolabs). A solution of 50 μg protein from cell lysate, 1 μL of 10× Glycoprotein Denaturing Buffer, and ddI H_2O at total volume of 10 μL was incubated for 10 min at 37°C. After incubation, 2 μL 10× G7 Buffer, 2 μL NP40, 2 μL PNGaseF enzyme and ddI H_2O were added to solutions to a new final volume of 20 μL. These solutions were incubated at 37°C for 72 hours and then directly loaded into precast gels for SDS-PAGE and immunoblot analysis.

Immunofluorescence Microscopy

Cells were grown in 6 well plates for 24–48 hours on sterile glass cover slips. Cover slips were gently washed twice with 1 mL of cold 1× PBS and fixed for 30 seconds with 1:1 Acetone:ddI H_2O. Cells were permeabilized and blocked with 5% milk in TBS-T (contains 0.1% TritonX100) for 1 hour at room temperature. Protein was labeled using primary monoclonal antibody for Ctr1 raised in rabbit and a green fluorescent dye-labeled secondary anti-rabbit antibody (Oregon Green® 488 goat anti-rabbit IgG (H+L)). Confocal immunofluorescence microscopy was performed using a Zeiss Axio Observer wide field fluorescence microscope and images were processed using MetaMorph 7.5 system software at the Duke University Light Microscope Core Facility.

Results

Model peptides of the first 14 amino acids of human Ctr1 (Ctr1-14) including several “mutant” peptides were synthesized by standard Fmoc solid-phase peptide synthesis and purified by HPLC. The wild-type sequence of Ctr1-14 is H_2N-MDHSHHMGMSYMDS. In order to determine which of the 3 His and 4 Met residues of Ctr1-14 are important for copper binding, each of these Met and His residues was systematically substituted for non metal-coordinating residues norleucine (Nle) or alanine (Ala), respectively. In the case of His-to-Ala or Met-to-Nle substitution, 2 lysines were added to the peptide carboxyl-terminus to increase water solubility. The peptide in which all 3 His are replaced by Ala is called HA, whereas peptides with individual His replaced at positions 3, 5 or 6 are called H3A, H5A, and H6A, respectively, and the doubly substituted His 5 and His 6 both to Ala is designated H56A. The peptide labeled Ctr1-14(MNle) refers to the sequence where all 4 Met residues are replaced by Nle while all His residues remain wild type, whereas MNleH56A contains all Met-to-Nle replacements in addition to replacement of both His 5 and 6 with Ala. Finally, Ac-Ctr1-14 contains the native sequence but lacks a free amino-terminal amine due

its acetyl cap. The names and sequences of all the peptides used in this study are shown in Table 4, along with their apparent or relative affinities for Cu(I) and Cu(II), which were determined as described in the following sections.

Amino-Terminal Model Peptides of Ctr1 Bind Cu(II) with High Affinity via an ATCUN Site

Visible spectra of solutions of peptide and Cu(II) in HEPES buffer at pH 7.4 are shown in Figure 2. The native sequence model peptide (Ctr1-14) plus 1 equivalent of $Cu(ClO_4)_2$ shows an absorbance maximum near 525 nm, typical of an albumin-like distorted square planar N,N^-,N^-,N coordination environment with Cu(II).[31] The spectrum is nearly identical to that of Cu(II)HSA (data not shown). Substitution of either all Met-to-Nle (MNle) or His at the fifth (H5A) or sixth position (H6A) caused no significant change in the peptide–Cu(II) spectrum (Figure 2), indicating that none of the Met, nor His in the fifth or sixth positions are coordinated to Cu(II) in the native peptide high affinity binding site. In contrast, alteration of His in the third position from the amino-terminus in the H3A peptide results in an absorbance shift, indicating that the coordination environment is different in this mutant peptide compared to the parent Ctr1-14. In addition, when the amino-terminal amine is capped by an acetyl group, the λ_{max} shifts to a longer wavelength indicating a change in coordination environment compared to the wild type sequence. These data are consistent with albumin-type coordination by the ATCUN site in Ctr1-14 in which the Cu(II) is coordinated by the imidazole nitrogen, two deprotonated amide nitrogens, and the terminal amine.

Apparent affinity constants at pH 7.4 in HEPES buffer were determined by using NTA as a competitive ligand. Solutions of 1:1 P:Cu(II) were titrated with a total of at least 2 equivalents of NTA and reactions monitored by UV-Vis spectroscopy, as shown by a representative example in Figure 3. Titrations were also done in the reverse direction. Binding constants for each model peptide are listed in Table 3.

Copper(II) binds to Ctr1-14 with an apparent log $K_{PCu(II)}$ of 11.0 ± 0.3 in 50 mM HEPES buffered at pH 7.4. HEPES is known to interact with Cu(II)[48] and is known to form a ternary species with the Cu(II)NTA complex.[32] A correction factor has been reported for the calculation of conditional affinity constants from apparent constants determined in the presence of HEPES buffer.[48] However, to our knowledge a similarly rigorous study has not been done for the case where HEPES participates in ternary species formation. We report here the apparent values for formation of peptide-Cu(II) complexes at pH 7.4 in the presence of 50 mM HEPES. These apparent values are uncorrected for the interaction of HEPES with Cu(II) and Cu(II)NTA and therefore the actual conditional values at pH 7.4 are likely to be higher. This strong binding to Cu(II) is comparable to the reported apparent value of human serum albumin (HSA) under similar buffer conditions (log $K_{HSACu(II)}$= 11.4 at pH 7.4 in 100 mM HEPES).[32] The similarity in the Cu(II) binding constants between HSA and the Ctr1-14 model peptide suggests that human Ctr1 may be able to compete with serum proteins such as albumin for Cu(II).

Substitution of all Met (MNle) and/or the His in the fifth and sixth positions (MNleH56A, H5A, H6A, and H56A) does not significantly affect peptide affinity for Cu(II). Only by removal of His in the third position from the amino-terminus (H3A and HA) or by acetyl-capping the amino-terminal amine (AcHCtr1-14) is a significant change in binding affinity for Cu(II) detected, as shown by a comparison of the values in Table 3. The H3A peptide was unique in that during the course of the spectrophotometric titration where peptide was titrated into a solution of 1:1 Cu(II):NTA, an unidentified species was formed with a λ_{max} = 680 nm (data not shown). This species is likely a ternary complex involving the peptide, Cu(II) and perhaps HEPES, or NTA. Due to the unidentified ternary complex, the data for H3A could not be fit to a simple model. Although the binding affinity was not calculated in

this case, it is clear that the peptide had significantly lower affinity for Cu(II) than other peptides. These results demonstrate that the high-affinity site for Cu(II) is the ATCUN amino-terminal unit provided by H_2N-MDH.

Human Ctr1-14 High Affinity Cu(I) Binding Requires Histidine

We have previously reported apparent dissociation constants of Ctr1 Mets motif peptides with Cu(I) to be in the μM range (log K~6) by using an ascorbic acid oxidation assay along with an electrospray ionization mass spectrometry method.[27,28] These methods are not applicable to studying metal binding to histidine because in the presence of excess ascorbic acid, H^+ ions compete with metal binding. Instead, a solution competition study was employed with the colorimetric Cu(I) indicator bicinchoninate (BCA) as a competitive ligand for Cu(I).

Solutions of $Cu(I)(BCA)_2$ were titrated with up to 50 equivalents of peptide and monitored spectrophotometrically, as shown in Figure 4. In these experiments a large excess of peptide ensures that a 1:1 P:Cu(I) species is present, even in the case where the peptide may have multiple potential Cu(I) binding sites. Assuming a 1:1 P:Cu(I) complex, the $K_{PCu(I)}$ values were determined and are shown in Table 3. The overall formation constant log β_2 for formation of $Cu(I)(BCA)_2$ has been reported by two independent sources providing two values that differ by 2.5 orders of magnitude (14.7 versus 17.3).[49,51] The former was obtained by isothermal titration calorimetry[51] and it has been argued that this lower value may be slightly inaccurate due to potential and unaccounted-for diminished heat release due to conversion of $Cu(I)Cl_n$ species to $Cu(I)(BCA)_2$.[50] The latter value was obtained via indirect competition for Cu(I) between ligands BCA and bathocuproine disulfonate (BCA, log β_2 = 19.8) mediated separately by three *apo* proteins that bind Cu(I) with different affinities.[49] In this work we considered both published values and report the results in Table 3 of using each in our equilibrium models in Table 2. Here only the lower values are discussed, as they provide a lower-limit for copper affinity.

An example of titration spectra taken upon addition of Ctr1-14 into a solution of BCA and Cu(I) is shown in Figure 4, where competition can be observed by the decrease of absorbance at 358 and 562 nm due to the $Cu(I)(BCA)_2$ complex as peptide is added to solution. Of the series of peptides tested, the strongest binding is observed from the Ctr1-14 native sequence, with an apparent $\log K_{PCu(I)}$ of 10.2(2), which corresponds to a dissociation constant (K_D) between 40–100 pM.

In contrast, substitution of all three His for Ala (HA peptide) renders the model peptide unable to compete for Cu(I) from the $Cu(I)(BCA)_2$ complex even with 50 equivalents of excess peptide. Previous studies on the Mets motif MGMSYM of human Ctr1-14 found that this peptide has a K_D for Cu(I) at pH 7.4 of 9 μM, which is too weak to be competitive with BCA.[28] According to the current model for Ctr1 function in vivo, the histidines in hCtr1-14 have no clear role in Cu(I) acquisition. However, the current experiment demonstrates that histidines contribute to strong Cu(I) binding affinity in a Ctr1 model peptide that is distinct from a low-affinity Mets motif binding site (~micromolar affinity) and suggests the possibility that histidines may contribute to Ctr1 Cu(I) acquisition.

Replacement of all methionine residues with norleucine has little if any effect on the ~70 pM affinity of the peptide for Cu(I). This supports evidence that His residues, rather than Mets motifs, are important for high affinity Cu(I) binding to Ctr1 model peptides at the physiological pH of 7.4.

Individual His substitution has a less drastic effect on the ability of peptides to compete for Cu(I) from BCA as compared to the all His-to-Ala substituted peptide. Replacement of the

histidines in the fifth or sixth position has little if any effect, whereas replacing both of these His together (H56A) reduces the binding constant by about one order of magnitude. Individual replacement of the histidine in the third position (H3A) also reduces the binding constant by an order of magnitude. These results indicate that all three His have some part in a Cu(I) binding site, but perhaps the fifth and sixth histidine are exchangeable and/or have a cumulative contribution to Cu(I) complex formation.

Based on the complete loss of Cu(I) competition with BCA in the MNleH56A peptide containing only the third position His, it is clear that Cu(I) does not bind strongly in the same way that Cu(II) does to the ATCUN site alone. This is not surprising because the soft Cu(I) would disfavor the harder donor character of amide and amine nitrogens according to Pearson's HSAB Principle.[55] The third position His seems to contribute to Cu(I) binding in a His-only or mixed His/Met donor environment. One possibility for coordination involving the fifth and sixth His is the type of linear two-coordinate bis-His site recently characterized by Karlin and Shearer and shown to possess unique redox chemistry in the presence of other donors.[37–40] The fifth and sixth His alone, however, are clearly not the only important residues contributing to a high-affinity Cu(I) binding site (Table 3).

HH Sequence Facilitates the Ascorbate-Dependent Reduction of Cu(II)-Peptide Complexes

It is clear from experiments discussed above that Ctr1 model peptides can bind both oxidation states of copper with significant affinity. It is especially noteworthy that the histidine residues are crucial for the model peptide's ability to compete with BCA for Cu(I) and NTA for Cu(II). Mets motifs alone are not sufficient for the high affinity binding necessary to compete for either oxidation state of copper. Because these peptides are capable of strong binding to both oxidation states of copper it would be interesting to acquire the reduction potentials of the PCu(II) complexes using cyclic voltammetry. The work of both Karlin and Shearer suggests that the presence of an HH domain might impart interesting redox properties to these peptides.[37–40] Unfortunately, attempts to measure reduction potentials via standard cyclic voltammetry protocols failed to produce a measurable signal. This is likely due to low diffusion coefficients or insulation of the Cu ion by the peptide.

Instead, differences in the ability of ascorbate to reduce Cu(II) complexes of the various peptides were measured in the presence of HEPES buffer at pH 7.4. The experiments were conducted under aerobic conditions where availability of oxygen would allow cycling of copper oxidation states (i.e. after being reduced by ascorbate, the Cu(I) could be re-oxidized by O_2 from the air). However, if the peptides are able to stabilize Cu(I) then the re-oxidation to Cu(II) will be slowed and Cu(I) complex should accumulate. In fact, this seems to be the case with some of the peptides and clear differences can be noted depending on the presence of the fifth and sixth position His (Figure 5).

In previous work by Perrone et al., the ascorbate-dependent reduction of Cu(II) complex with HSA and a model peptide with the sequence, DAHK, based on the ATCUN motif of HSA, were tested via a similar procedure under anaerobic conditions.[56] This previous work found that the Cu(II) complex of a peptide with the sequence DAHK was stable in the presence of ascorbic acid whereas HSA complex with Cu(II) was reduced to a Cu(I) species. The copper reduction could be monitored by a decrease of the Cu(II) HSA signal at 525 nm. We repeated this experiment with HSA under aerobic conditions and found comparable results under these conditions.

The Cu(II) complex of Ctr1-14 demonstrates a reduction in the Cu(II) signal over 1 hour after adding 2 equivalents of ascorbate to solution, indicating an ascorbate-dependent Cu(II) complex reduction to a Cu(I) complex. Similarly to Ctr1-14 and HSA, the MNle peptide Cu(II) complex could be reduced and assumingly stabilize the Cu(I) species under these

conditions. However, upon substitution of either His in the fifth or sixth position, the Cu(II) signal did not change over 1 hour with ascorbate. These data indicate that both His 5 and 6 are important for stabilizing the Cu(I) species and perhaps facilitating the reduction of the Cu(II) to Cu(I) complex by increasing the reduction potential.

Ctr1ntHA Localizes to the Plasma Membrane and is Functionally Compromised in Ctr1$^{-/-}$ MEF

Mouse embryonic fibroblast (MEF) cells, including wild type (wt), Ctr1 knockout (Ctr1$^{-/-}$) and, Ctr1 reconstituted stable cell lines were generated previously.[53] A vector containing cDNA for a human Ctr1 mutant with all amino-terminal His mutated to Ala (Ctr1ntHA) was generated and stably expressed in Ctr1$^{-/-}$ cells at levels comparable to the wild type Ctr1 protein.

Immunofluorescence microscopy was employed to determine the localization status of the Ctr1ntHA mutant in MEF cells. Cells were fixed and permeabilized in 1:1 MeOH:Acetone and probed with an anti-Ctr1 primary antibody, previously described.[54,57] In short, it is an antibody against a synthetic 18-mer peptide of the sequence H_2N-VSIRYNSMPVPGPNGTILC-CO_2H, which corresponds to the cytosolic loop between transmembrane domains 1 and 2 of mouse and human Ctr1. Secondary antibody covalently linked to a green fluorescent dye was used to visualize Ctr1 and assess its location in the cells.

Cells expressing Ctr1ntHA mutant show Ctr1 localization similar to wild type (not shown) and Ctr1$^{+/+}$ cells. This is not surprising based on previous findings that the amino-terminal domain of hCtr1 does not contain signals required for protein trafficking to the plasma membrane.[21,23,29,58,59]

Stable cell lines expressing Ctr1ntHA were evaluated by immunoblotting for expression levels of Ctr1 and CCS, the Cu chaperone for Cu, Zn Superoxide dismutase. CCS serves as an indicator for cellular copper status, as its levels are elevated under copper deficiency and reduced when copper levels are high due to copper-dependent proteasomal turnover.[60] The intensity of CCS was quantified and normalized to actin, a loading control (Figure 7).

In the immunoblot shown in Figure 7a and the histogram shown in Figure 7b, Ctr1$^{-/-}$ MEF stably expressing Ctr1 or Ctr1ntHA express several polypeptide species that are recognized by the specific Ctr1 primary antibody. In the literature, reported patterns of polypeptide species corresponding to Ctr1 vary significantly depending on the antibody used, the cell type and the Ctr1 species. Although predicted molecular mass of the Ctr1 primary translation product is 21 kDa, the size of the protein on western blots has been reported as 17, 24, 28, 35, and 37 kDa, as well as multimeric forms of higher molecular mass.[57,58] The lowest band of ~17 KDa is attributed to an amino-terminally cleaved Ctr1 monomer, while the higher group of bands between about 24 and 37 KDa can be attributed to heterogeneous N-linked and O-linked glycosylation states.[30,58]

Cells expressing Ctr1ntHA show higher CCS levels compared to the wild type Ctr1-reconstituted control cells. The data shown are representative of several independent experiments using independently derived Ctr$^{-/-}$ MEF stably expressing Ctr1ntHA (data not shown) and suggest that the cells expressing the Ctr1ntHA mutant have lower copper levels than cells expressing wild type Ctr1.

As could be expected from a Ctr1 mutant that is compromised in copper transport function, Ctr1ntHA expression results in a phenotype consistent with decreased intracellular copper status. Interestingly, we also observe the pattern of Ctr1 bands between 25 and 37 KDa

shows a unique electrophoretic profile for cells expressing Ctr1ntHA compared to cells expressing wild type Ctr1. Ctr1 is heterogeneously glycosylated at threonine-27 and asparagine-15. Recent evidence has shown that O-linked glycosylation at threonine-27, but not N-linked glycosylation at asparagine-15, protects against truncation of the Ctr1 amino-terminus and prevents impaired copper transport by the cleaved form.[9,58] If the mutation of His to Ala in our mutant somehow prevented O-glycosylation, we would expect to see a single band at 17 KDa corresponding to the truncated protein. However, we see major bands between 24–37 kDa in the Ctr1ntHA mutant suggesting that the protein is intact and retains O-glycosylation. We tested the mutant Ctr1 protein for N-linked glycosylation and found that the Ctr1ntHA protein is also glycosylated by N-linked sugars, most likely at asparagine-15, as expected from normal processing of the Ctr1 (Figure S1). We also considered that the variation in band pattern could be due to variations in multimer formation, however the consistency of band pattern under reducing and non-reducing conditions suggests that the Ctr1ntHA is not forming unusual multimers compared to Ctr1 (Figure S2). While we do not have a clear understanding of why Ctr1 and Ctr1ntHA show different band patterns between 24–37 kDa, our data indicate that the cell is processing Ctr1ntHA protein properly. The differences we observe may be due to simple change in electrophoretic mobility based on sequence change, or may be due to an altered glycosylation profile that should not affect Ctr1 function. Based on our evidence that model peptides of human Ctr1 bind more strongly to Cu(I) and Cu(II) via His compared to Met alone, it is possible that the copper deficiency observed here is due to lack of strong copper binding to extracellular Ctr1 histidine residues at physiological pH.

Discussion

The current model for how mammalian cells acquire copper from their extracellular environment with high affinity is that the cell surface copper transporter, Ctr1, imports Cu(I) and does so primarily via interactions between Cu(I) and methionine-rich Mets motifs.[4–9,21–23,59] Presented here is evidence that histidine residues in model peptides from the extracellular amino-terminus of human Ctr1 have significant affinity for copper in both oxidation states of Cu(I) and Cu(II), which suggests the possibility that histidine ligands may play a previously underappreciated role in mammalian copper acquisition. In addition we show, in the context of intact Ctr1, evidence that mutation of the histidine residues contributes to a phenotype indicative of copper deficiency.

For the purposes of this discussion “high affinity” sites refer to those binding Cu(I) with stronger than nanomolar affinity (log K_a~9 or greater) or Cu(II) with tighter than 10 picomolar affinity (log K_a~11). “Low affinity” sites refer to anything with affinity two orders of magnitude or more reduced from high affinity sites. The presence of an ATCUN site at the amino-terminus allows Ctr1 model peptides to bind Cu(II) with 10 picomolar affinity at pH 7.4 (log $K_{PCu(II)}$~11). This high affinity for Cu(II) should allow the Ctr1 protein to compete with biologically relevant extracellular copper carriers, like HSA. This Cu(II) interaction has potential importance in understanding the mechanism for Ctr1 copper acquisition. Specifically, the high binding affinity of Ctr1 model peptides for Cu(II) implies that in vivo the protein may be occupied by Cu(II) at the ATCUN site prior to copper reduction by a physiological reducing agent.

The presence of histidine residues also significantly increases Ctr1 model peptide affinity for Cu(I) compared to methionine-only motifs. The high affinity Cu(I) sites identified here contain at least an HH motif, in addition to which additional His or Met ligands could provide greater affinity to give subnanomolar binding for Cu(I). In contrast, $MX_{1-2}MX_{1-2}M$ motifs bind Cu(I) with micromolar affinity,[27,28] which classifies them here as lower-affinity sites.

The experimental results presented here also show that the HH site in these Ctr1 model peptides affects the redox properties of the peptide-Cu(II) complexes. This apparent facilitation of Cu(II) reduction or simple stabilization of Cu(I) in the case of peptides containing the HH site has significant implications for the in vivo function of this protein. The similar reactivity between Ctr1 and HSA is also interesting. We suggest that perhaps the ascorbate present in human serum (30–150 μM)[61] could function as a reductant for Cu(II) at the plasma membrane in the presence of either HSA or Ctr1. This facilitation of Cu(II) reduction in the presence of both Ctr1 and HSA could be an important clue to understanding the mechanism for reduction of Cu(II) at the plasma membrane and for understanding the mechanism of copper acquisition and transport via Ctr1. In either case, it is clear that His may have a functional role in Ctr1-mediated copper acquisition by imparting special redox properties on the peptide-copper complex.

The results from these model peptide studies have been extended to live cell culture studies and results are consistent with our hypothesis that His residues play a previously underappreciated role in copper transport. $Ctr1^{-/-}$ MEF expressing Ctr1ntHA display high CCS levels compared to $Ctr1^{-/-}$ MEF expressing wild type Ctr1. The high CCS levels of cells expressing Ctr1ntHA suggest that these cells have an intermediate copper deficiency between wild type and $Ctr1^{-/-}$ MEF. In light of results obtained with model peptides, it is likely that Ctr1ntHA is inefficient at recruiting copper to the extracellular pore or that it is inefficient in facilitating copper reduction in the presence of weak extracellular reducing agents via a direct copper ligand function of the histidine residues.

Our present findings are not consistent with studies of hCtr1 in yeast, HEK293 and SF9 cells reported previously that found no significant phenotype for cells expressing histidine mutants of Ctr1.[21,23] These studies however were either done in yeast cells, or were done by overexpressing transgenic Ctr1 mutant protein with an amino-terminal myc or FLAG epitope tag in a system expressing endogenous native Ctr1. Amino-terminal tagging of Ctr1 blocks potential high affinity Cu(II) binding to the ATCUN site, while detection of a phenotype in yeast ctr1Δ cells or mammalian cells expressing a background of endogenous Ctr1 would make the system less sensitive to functional perturbations. In the results presented here, detection of a copper deficient phenotype for cells expressing Ctr1ntHA is likely due to the increased sensitivity of the methods used, particularly by employing a $Ctr1^{-/-}$ mammalian cell line at a pH above 6 and the use of untagged Ctr1 alleles.

The possibility that Ctr1 amino-terminal His residues are important for copper acquisition may have important implications with respect to the current description for copper transport in mammalian cells. Current models suggest that Ctr1 Mets motifs facilitate extracellular copper acquisition, and that an unidentified cell surface reductase is necessary for copper reduction. Here we suggest potential Ctr1 competition with extracellular proteins for copper binding. In addition, evidence presented here suggests that albumin or Ctr1 itself might have the ability to facilitate copper reduction in the presence of physiological reducing agents such as ascorbate. This model for copper acquisition via mammalian Ctr1 is distinct from the model for yeast copper acquisition in that mammalian Ctr1 at pH 7.4 might employ histidine as ligands for Ctr1 extracellular copper acquisition, whereas in yeast, extracellular milieu below pH 6 prevents histidine from being an efficient copper ligand. Although the His clusters in human Ctr1 are more efficient at binding copper at high pH, in the case of yeast, methionines are ideal ligands for copper recruitment. We have shown recently that copper binding to Mets motifs is independent of pH, whereas peptides with both His and Met residues demonstrate pH-dependent copper binding, with all-Mets motifs being more efficient than Met/His combinations at pH 4.5.[62] Humans may need multifunctional Ctr1 proteins that can operate at multiple pH values, found in distinct tissues, and that can compete with copper carriers found in extracellular fluids. In humans, dietary copper

absorption likely occurs via all three sections of the intestine, where the pH ranges from pH 5 in the duodenum, pH 6–7 in the jejunum, and pH 7–7.3 in the ileum, while transport of copper from plasma to cells in peripheral tissues occurs at or near pH 7.4. It is possible that mammalian Ctr1 utilizes a combination of His clusters and Mets motifs to accomplish copper acquisition under these different cell and tissue-specific scenarios.

The findings here call for further investigation of the role of extracellular histidine ligands in Ctr1-mediated copper acquisition and transport. Consistent with this is recent evidence demonstrating that Ctr1 lacking its His-rich extracellular domain due to truncation of ~30 amino-terminal amino acids transports ^{64}Cu at only 50–60% efficiency compared to the intact Ctr1 protein.[30] This result cannot be explained by the current model of Ctr1 Cu-binding function, but can be explained based on the data here demonstrating that the Ctr1 amino-terminal model peptide His residues can participate in strong Cu(I) and Cu(II) binding at pH 7.4 and are likely to facilitate Ctr1-mediated Cu acquisition.

The potential physiological significance of ATCUN and other His residues in the amino-terminal domain of mammalian Ctr1 is not yet fully understood. We still do not know whether the ATCUN site alone is important for copper acquisition and whether Ctr1 uses this site to compete for copper from extracellular copper carriers such as HSA and transcuprein. Recent evidence suggests that at least three mechanisms may be responsible for cellular copper uptake and that transcuprein, HSA, and perhaps other copper complexes of amino acids, may each play unique roles in delivering copper to Ctr1 or other cellular copper transport machinery, and that the mechanisms of copper delivery and uptake may vary with cell type.[63] Here we demonstrate a new role for Ctr1 amino-terminal histidine clusters in facilitation of copper reduction and cellular copper acquisition. We do not fully understand the coordination of Cu(I) and the role of Ctr1 or HSA in facilitation of copper reduction, delivery, and uptake, however, this work provides insight into possible mechanisms of Ctr1-dependent cellular copper acquisition and the potential role of histidines in Ctr1 function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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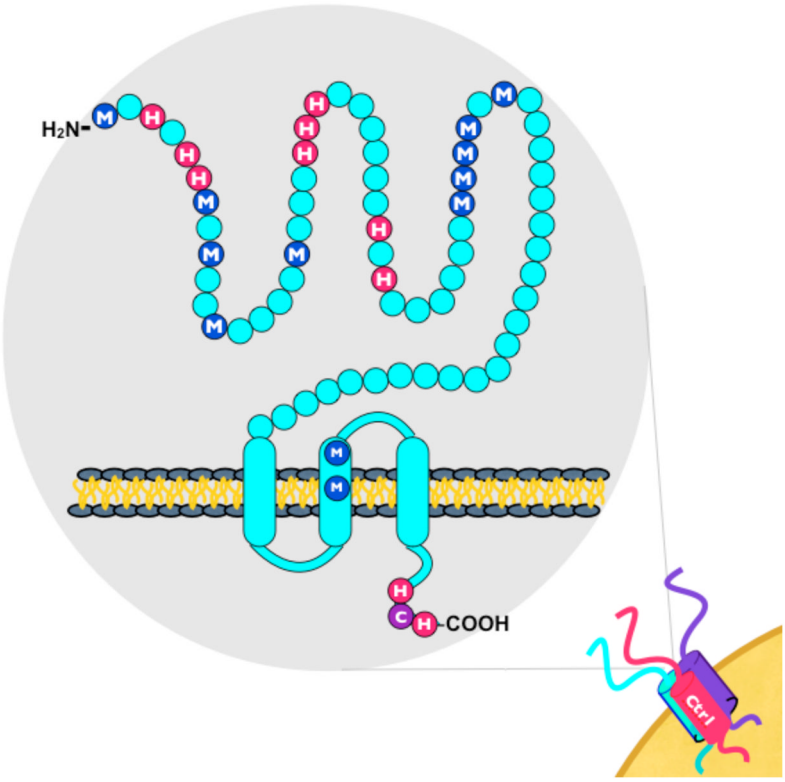

Figure 1.
Human Ctr1 assembles as a homotrimer in the plasma membrane with an amino-terminal extracellular domain equipped with His (H) and Met (M) potential metal-binding residues. All extracellular His and Met residues are indicated. The functionally critical TMD2 methionine residues and carboxyl-terminal cysteine and histidine residues are indicated.

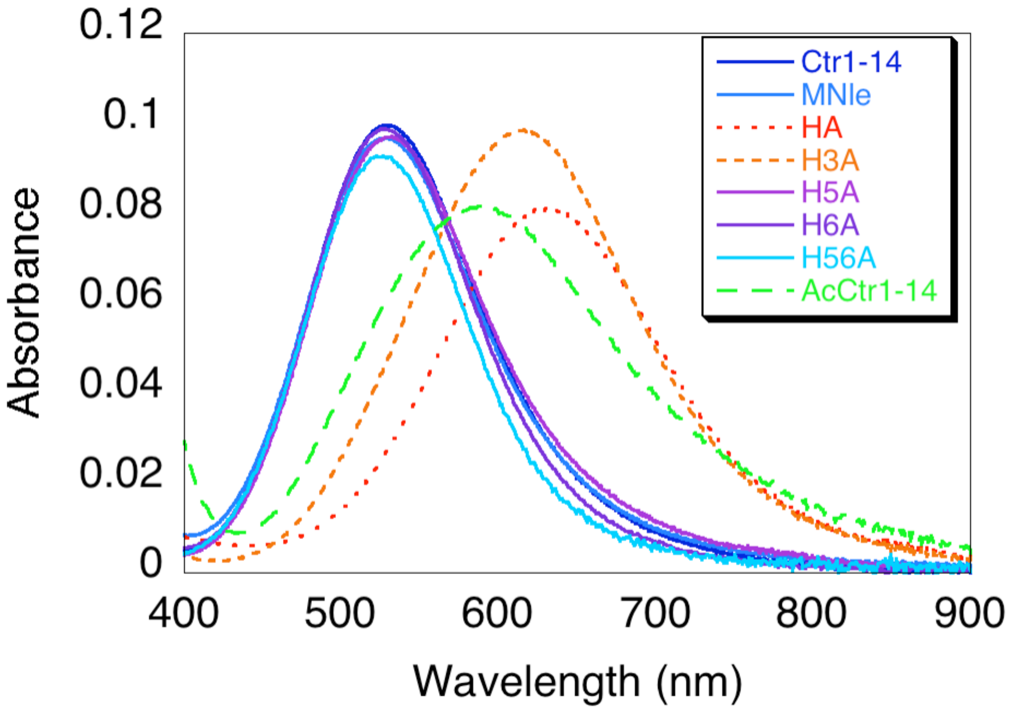

Figure 2.

Absorbance spectra of Ctr1 model peptides (~500 μM) with 1 equivalent of Cu(II) at pH 7.4 in HEPES buffer. Peptides with His in the third position from the amino-terminus display absorbance at 525 nm characteristic of a typical ATCUN Cu(II) binding site. Substitution of all His-to-Ala, individual substitution of the His at position 3 (H3A) or acetyl capping of the N-terminus (Ac-Ctr1-14) results in a different type of binding site and a shift in λ_{max} compared to the wild-type peptide (Ctr1-14).

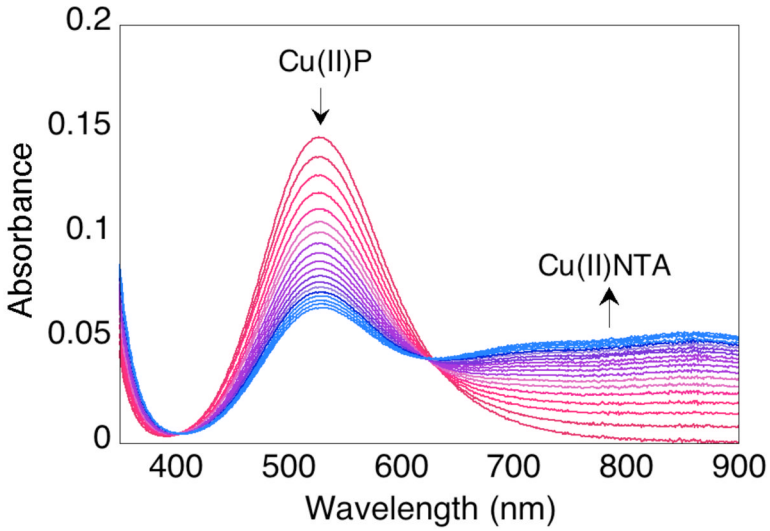

Figure 3.
Visible spectra showing the results of a titration of NTA into a 500 μM solution of 1:1 P:Cu(II) in 50 mM HEPES buffer at pH 7.4, where P = Ctr1-14.

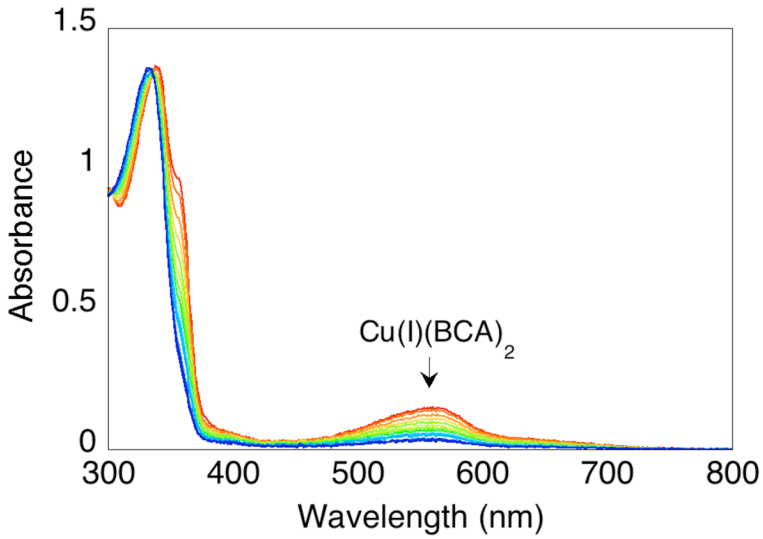

Figure 4.

Spectra from a titration of 50 equivalents of Ctr1-14 peptide into 75 μM BCA and 25 μM $[Cu(I)(CH_3CN)_4]PF_6$ in 50 mM HEPES buffer at pH 7.4 and 0.5% CH_3CN.

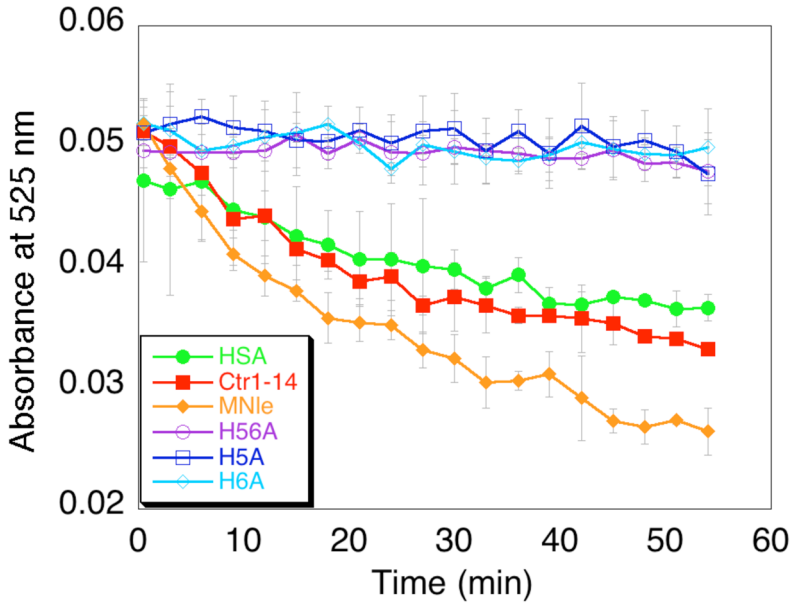

Figure 5.

Ascorbate-dependent reduction of Cu(II) in complex with Ctr1-14 model peptides containing the ATCUN site. Solutions of 500 μM peptide-Cu(II) complex and 1 mM ascorbate in 50 mM HEPES at pH 7.4 monitored for 1 hour with UV-Vis at 525 nm, the characteristic absorbance band due to the Cu(II) complex.

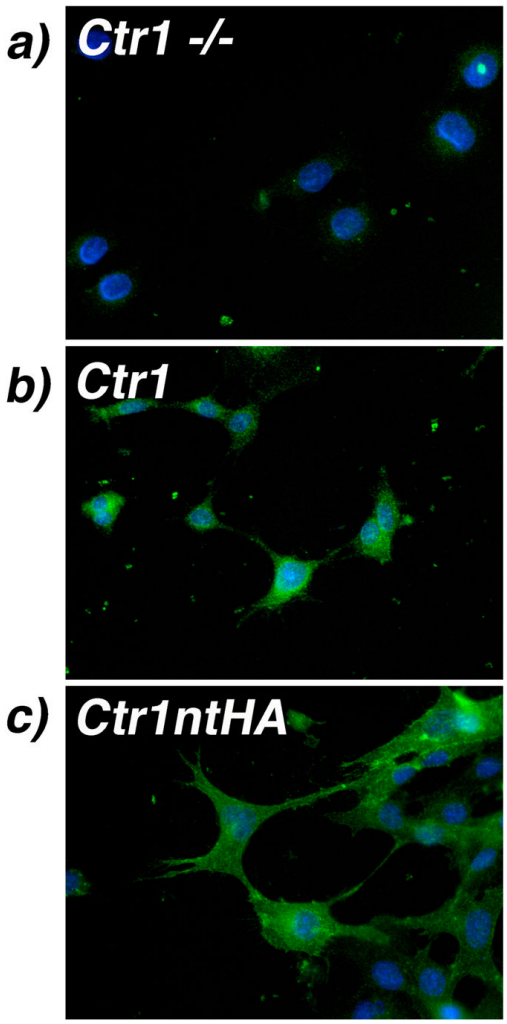

Figure 6.

Immunofluorescent imaging of Ctr1 expression in various cell lines. Fixed cells were probed with specific primary antibody for Ctr1 and secondary antibody fused to a green fluorescent dye. Slides were mounted with DAPI stain to visualize the nucleus (blue). a) MEF $Ctr1^{-/-}$ cells with both alleles of Ctr1 deleted do not express Ctr1 and show only low background fluorescence. b) MEF $Ctr1^{-/-}$ cell line stably expressing Ctr1. General distribution of green fluorescence is typical for normally-functioning Ctr1, which localizes to the plasma membrane as well as endosomes. c) MEF $Ctr1^{-/-}$ cell line stably expressing Ctr1ntHA mutant shows typical distribution expected for Ctr1 localized to the plasma membrane and endosomes.

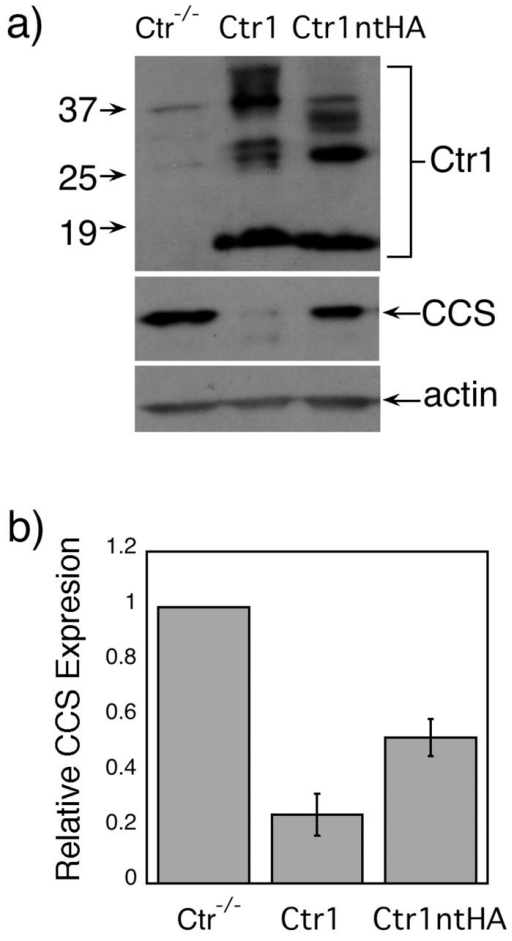

Figure 7.

High CCS levels in Ctr1ntHA expressing cells indicate a copper deficient phenotype. a) Immunoblot showing expression levels of proteins recognized by specific antibodies against Ctr1, CCS and actin in Ctr1 knockout MEF cells ($Ctr1^{-/-}$), $Ctr1^{-/-}$ cells stably expressing transgenic human wild type Ctr1 (Ctr1) or the amino-terminal Ctr1 His to Ala mutant (Ctr1ntHA). Cells expressing Ctr1ntHA have high CCS levels compared to the Ctr1 transgenic control. High CCS levels in cells expressing Ctr1ntHA indicate that His mutation causes an intracellular copper deficiency. b) CCS levels quantified over three experiments using an individual stable cell line expressing Ctr1ntHA. Data were quantified using Photoshop software. Band intensities from CCS were normalized to band intensities from an actin loading control. Data from separate experiments were compared by normalizing all data to the relative intensity of CCS expression levels of $Ctr1^{-/-}$ cells from each experiment.

Table 1

Model used for the competition of Ctr1 model peptides and NTA for Cu^{2+} in HEPES buffer at pH 7.4. The value for log β_1 obtained by this model for CuP is an apparent constant

Species	Log βlmh	Cu m	NTA l	P l	H h	
CuP		1	0	1	0	Refined
Cu(NTA)	12.7	1	1	0	0	Constant[47]
$Cu(NTA)_2$	17.4	1	2	0	0	Constant[47]
NTAH	9.46	0	1	0	1	Constant[47]
$NTAH_2$	11.95	0	1	0	2	Constant[47]
$NTAH_3$	13.76	0	1	0	3	Constant[47]
$NTAH_4$	14.76	0	1	0	4	Constant[47]
CuOH	−8.2	1	0	0	−1	Constant[47]

Table 2

Model used for the competition of Ctr1 model peptides and BCA for Cu(I) in HEPES buffer at pH 7.4 and 0.5% acetonitrile. The log β_1 solved by this model for CuP is an apparent constant

a) Model based on data from Ref 51

Species	Log β	Cu	NTA	P	H	
		m	*l*	*l*	*h*	
CuP		1	0	1	0	Refined
Cu(BCA)	7.3	1	1	0	0	Constant[51]
$Cu(BCA)_2$	14.7	1	2	0	0	Constant[51]

b) Model based on Ref 49

Species	Log β	Cu	NTA	P	H	
		m	*l*	*l*	*h*	
CuP		1	0	1	0	Refined
$Cu(BCA)_2$	17.3	1	2	0	0	Constant[49]

Table 3

DNA mutagenesis and amplification primers

Primer/Set	Sequence
Ctr1H2A-F	5′-ATGGACTCCAACAGTACCATGCAACCTTCTGCCGCTGCCCCAACCACTTCAGCC-3′
Ctr1H2A-R	5′-GGCTGAAGTGGTTGGGGCAGCGGCAGAAGGTTGCATGGTACTGTTGGAGTCCAT-3′
Ctr1H3A-F	5′-CACCCAACCACTTCAGCCTCAGCCTCCGCTGGTGGAGGAG-3′
Ctr1H3A-R	5′-CTCCTCCACCAGCGGAGGCTGAGGCTGAAGTGGTTGGGTG-3′
Ctr1HA_EcoR1-F	5′-ATATGAATTCATGGATGCCTCCGCCTCCGCCGCCAT-3′
Ctr1HA_XhoI-R	5′-ATATCTCGAGTCAATGGCAATGCTCTGTGATATCC-3′

Table 4

Names and sequences of Ctr1 model peptides together with experimentally determined Cu(I) and Cu(II) apparent binding constants at pH 7.4. All peptides contain a carboxyl-terminal amide and amino-terminal amine, except for Ac-Ctr1-14, which contains an amino-terminal acetyl cap, as indicated

Peptide Name	Amino Acid Sequence	log K Cu(I) using model in Table 2a	log K Cu(I) using model in Table 2b	log K Cu(II) from Table 1 model
Ctr1-14	M D H S H H M G M S Y M D S	10.2 ± 0.2	12.8 ± 0.1	11.0 ± 0.3
HA	M D A S A A M G M S Y M D S K K	*	*	5.0 ± 0.7
H3A	M D A S H H M G M S Y M D S K K	9.1 ± 0.1	11.7 ± 0.1	**
H5A	M D H S A H M G M S Y M D S K K	10.01 ± 0.09	12.61 ± 0.09	11.3 ± 0.3
H6A	M D H S H A M G M S Y M D S K K	9.8 ± 0.1	12.4 ± 0.1	11.2 ± 0.1
H56A	M D H S A A M G M S Y M D S K K	8.86 ± 0.03	11.46 ± 0.03	10.87 ± 0.06
MNleH56A	Nle D H S A A Nle G Nle S Y Nle D S K K	*	*	11.29 ± 0.07
MNle	Nle D H S H H Nle G Nle S Y Nle D S K K	9.55 ± 0.04	12.13 ± 0.04	10.6 ± 0.2
Ac-Ctr1-14	Ac-M D H S H H M G M S Y M D S			9.12 ± 0.04

Metal binding residues are highlighted. Magenta, histidine. Blue, methionine. N-terminal amine nitrogen not shown although it is a Cu(II) binding donor atom. “Ac” indicates an acetylated N-terminus.

* Peptide does not compete with BCA for Cu(I).

** Data does not fit model, peptide forms ternary species with Cu(II) and HEPES or NTA