The Mechanism of Copper Uptake Mediated by Human CTR1
A MUTATIONAL ANALYSIS

Received for publication, August 10, 2005, and in revised form, August 29, 2005. Published, JBC Papers in Press, August 31, 2005. DOI 10.1074/jbc.M508822200

John F. Eisses and Jack H. Kaplan
From the Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, Illinois 60607

Cellular copper uptake is a prerequisite for the biosynthesis of many copper-dependent enzymes; disruption of copper uptake results in embryonic lethality. In humans, copper is transported into cells by hCTR1, a membrane protein, composed of 190 amino acids with only three trans-membrane segments. To provide insight into the mechanism of this unusual transporter, we characterized the functional properties of various hCTR1 mutants stably expressed in Sf9 cells. Most single amino acid substitutions involving charged and potential copper-coordinating residues have some influence on the V_{max} and K_{m} values for copper uptake but do not greatly alter hCTR1-mediated copper transport. However, there were two notable exceptions. Replacement of Tyr^{156} with Ala greatly reduced the maximal transport rate without effect on the K_{m} value for copper. Also, replacement of His^{139} in the second trans-membrane segment with Arg caused a dramatic increase in the rate of copper uptake and a large increase in the K_{m} value for copper. This effect was not seen with an Ala replacement, pointing to the role of a positive charge in modulating copper exit from the pathway. Truncated mutants demonstrated that the deletion of a large portion of the N-terminal domain only slightly decreased the apparent K_{m} value for copper and decreased the rate of transport. Similar effects were observed with the removal of the last 11 C-terminal residues. The results suggested that the N and C termini, although nonessential for transport, may have an important role in facilitating the delivery of copper to and retrieving copper from, respectively, the translocation pathway. A model of how hCTR1 mediates copper entry into cells was proposed that included a rate-limiting site in the pore close to the intracellular exit.

Copper is an essential element required for many important cellular reactions that rely on the redox properties of this metal (1–3). Like many of the essential trace metals, inappropriately high concentrations of copper are toxic, both at the cellular and whole organism level. Thus intracellular copper is tightly regulated so that sufficient amounts are absorbed and excess copper is efficiently excreted (2, 4–10). Copper homeostasis at the cellular level involves the entry and exit of copper as well as the processes whereby copper is delivered to specific subcellular locations. The regulatory mechanisms that govern the cellular localization of proteins involved in copper transport are currently under investigation, and some progress has been made in understanding these pathways (11–14). However, studies 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 nonspecific divalent metal ion transporter, is believed to play a role in copper(II) uptake in the small intestine (15), whereas CTR1, a more specific transporter for copper (and perhaps silver), is thought to mediate Cu(I) uptake in many tissues. The physiological importance of hCTR1 is demonstrated by its essential role in embryonic development (16, 17). Recent studies have also provided the first insights into structure-function relationships in hCTR1 (18, 19).

hCTR1 is a glycosylated trans-membrane protein, which consists of 190 amino acids and has three putative trans-membrane segments. The N terminus is extracellular and has a single N-linked glycosylation site at Asn^{15}, and the C terminus is localized within the cell (20). Recent studies demonstrated that mutations of the methionines in the N-terminal domain (Met^{40}–Met^{45}) and in the second trans-membrane segment (Met^{150} and Met^{154}) decrease the ability of hCTR1 to transport copper into cells (19). Complementation assays applied to a yeast homologue of hCTR1 (yCtr3) also demonstrated the structural importance of residues in the third trans-membrane segment. The glycine residues Gly^{167} and Gly^{171} in this segment have been suggested to contribute to helix packing interactions and oligomerization of hCTR1 to form trimers (18). Additionally, Aller et al. (18) used tryptophan scanning of the third trans-membrane segment of yCtr3 to provide evidence that other residues (Ile^{196}, Ile^{197}, Ser^{198}, Cys^{199}, and Arg^{205}) may have a role in protein folding and structural integrity. Because of the sequence homology between yCtr3 and hCTR1, these residues may also be relevant for hCTR1.

Although these initial studies provided first glimpses into the molecular organization of CTR1, the mechanism of copper translocation by this transporter remains uncharacterized. Are there specific copper-binding sites that are alternately exposed to the outside and the inside of the cell via protein conformational changes? Are there amino acid residues that control entry of copper into a translocation pathway? Does copper migrate through CTR1 via a chain of “essential” residues or is there a “channel-like” pathway through which copper migrates without forming strong contacts with the pore-lining amino acid residues? Does the exit of copper from the transporter represent a regulated event? What is the rate-limiting step of transport? The answers to these questions are currently unknown. To begin addressing these important mechanistic issues, it is essential to go beyond complementation studies and provide direct and quantitative analyses of hCTR1 transport function. Consequently, we have utilized stable overexpression of hCTR1 in insect cells and carried out measurements of radioisotopic copper

* This work was supported by National Institutes of Health Grant P01 GM067166 Copper Entry into Human Cells, Project 1 (to J. H. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Genetics, University of Illinois at Chicago, 900 S Ashland Ave., Chicago, IL 60607. Tel.: 312-355-2732; Fax: 312-355-1765; E-mail: kaplanj@uic.edu.

2 The abbreviations used are: DMT1, divalent metal transporter 1; Wt, wild type; h, human.
uptake over a range of copper concentrations for various hCTR1 mutants. The roles of the intramembrane charged residues, residues potentially involved in copper coordination, and both the N and the C terminus were characterized. We combine our results with those of previous studies to propose a model of how hCTR1 mediates copper entry into cells.

**EXPERIMENTAL PROCEDURES**

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) to ensure retention of the expression vector.

**Generation of Mutant hCTR1 Constructs**—hCTR1 constructs with various mutations and deletions were generated by PCR using the full-length hCTR1 cDNA as a template (20). The outside primers (5′-H11032 forward primer, 5′-H11032-GAATTCATGGATCATTCCC-3′/H11032 reverse primer, 5′-H11032-CCGCGGAACAACTTCCCACTGC-3′, which contain EcoRI and SacII restriction sites at the 5′ and 3′ ends, respectively) and internal primers specific for the mutation generated (TABLE ONE) were utilized in the Pfu polymerase (Stratagene, La Jolla, CA.)-mediated reaction for full-length constructs. Primers designed to generate truncation constructs were used with either the 5′ forward or 3′ reverse primers, respectively. The PCR products were ligated into pIB/H5 TOPO vector using the TOPO-cloning technology (Invitrogen). The two C-terminal truncations were PCR-amplified using a construct containing Asn15 mutated to glutamine (20). This enabled us to monitor expression of the constructs that lacked the epitope for the C-terminally directed antibody. For these constructs we had to utilize an antibody directed against the N terminus that recognizes only the unglycosylated form of hCTR1. Plasma membrane protein expression of N15Q is the same as the plasma membrane expression of Wt hCTR1 in this system. The presence of desired mutations was confirmed by sequencing the entire cDNA.

**Analysis of Protein Expression**—For protein expression, each pIB plasmid encoding the mutant hCTR1 was transfected into Sf9 cells using Cellfectin reagent following the manufacturer’s protocol. Cells were selected using 80 μg/ml blasticidin-HCl for 2 weeks. Each stable cell line was then examined for hCTR1 protein expression by Western blot analysis using antibody directed against the C terminus of hCTR1 or, in the case of the two C-terminal deletions, an antibody raised against the N-terminal peptide (SHHHPTTSASHHG). For these experiments, cells were collected and homogenized using a Dounce homogenizer. The “total” membrane preparations were fractionated using a sucrose gradient, as described previously (20). To visualize hCTR1, 20 μg of plasma membrane protein was resolved using a 12% SDS-PAGE Laemmli gel. The proteins were electroblotted to a nitrocel-
lulose membrane and blocked using phosphate-buffered saline, pH 7.4, containing 5% milk. After blocking, the membranes were incubated with anti-C-terminal antibody (1:25,000, phosphate-buffered saline, pH 7.4, 0.1% Tween 20, 0.5% milk), and detection was carried out using a secondary goat anti-rabbit antibody (1:10,000, phosphate-buffered saline, pH 7.4, 0.1% Tween 20, 0.5% milk). There was some variation in the plasma membrane abundance among the various mutant proteins. The plasma membrane expression levels in the stably expressing Sf9 cell lines were normalized with respect to the expression of wild type hCTR1. The normalization allowed a direct comparison of the V_max values among the various mutants. The quantitation was accomplished by densitometric determination of nonsaturated Western blots of the plasma membrane fractions using the Alpha Innotech FluorChem 5500 imaging system (San Leandro, CA). The V_max values for transport were then corrected by utilizing the normalized value for protein expression for the mutants compared with wild type hCTR1 expression. A notable feature of the proteins expressed in stable cell lines is the relative lower level of higher order multimers that are stable in SDS gels compared with virally infected cells (20).

Expression and Function of N-terminally Truncated hCTR1—It was hypothesized that Met- and His-rich sequences in the N-terminal domain of hCTR1 could be important for copper binding and concentrating metal prior to entry into the trans-membrane pathway. To examine the role of multiple Met residues and His residues in guiding copper toward the permeation pathway, we generated three N-terminal deletions of hCTR1, and we investigated the effect of these deletions on the copper uptake (see also TABLE TWO). In this figure we also show the hCTR1-dependent uptake that results from the subtraction of the copper uptake into untransfected cells from the uptake into cells expressing hCTR1. All subsequent data presented on the kinetics of copper uptake into Sf9 cells have been corrected by this subtraction procedure.

The stably expressed hCTR1 is functional, as evident from the kinetics of copper uptake into cells shown in Fig. 2. Specifically, expression of hCTR1 protein is accompanied by a large (about 5-fold) increase in copper uptake (see also TABLE TWO). In this figure we also show the hCTR1-dependent uptake that results from the subtraction of the copper uptake from Sf9 cells expressing hCTR1.

RESULTS

Stable Expression of hCTR1 in Sf9 Cells Improves Quantitative Measurements of Copper Uptake—The trans-membrane portion of CTR1 contains a number of amino acid residues that may critically contribute to the translocation of Cu(I) across the membrane (Fig. 1). These include residues that could be involved in copper coordination, such as Cys, His, and Met, as well as negatively charged and polar residues (Asp, Glu, and Tyr). In addition, both the C terminus and especially the N terminus contain copper-coordinating residues with only a poorly defined role in copper uptake. To investigate the functional role of these various amino acids, we generated the appropriate mutations and truncations and stably expressed these proteins as well as wild type hCTR1 in Sf9 cells. Stable expression was expected to facilitate comparative analysis of mutants, because variations in virus titer, unavoidable in the transient transfection experiments, can be eliminated. Because functional expression of hCTR1 in stably transfected cells has not been described previously, we first characterized the properties of wild type hCTR1 in these cells.

Fig. 2 (inset) illustrates the distribution of wild type hCTR1 in membrane fractions of Sf9 cells. The stably expressed hCTR1 is present predominantly in the plasma membrane fraction consistent with its function and localization reported previously in mammalian cells (14). Two protein bands are observed following immunostaining with anti-hCTR1 antibody, a 28-kDa band corresponding to glycosylated hCTR1 monomer, and a 24-kDa band that is unglycosylated hCTR1. In some membrane preparations a C-terminal degradation product of 17 kDa is also seen. No protein bands are seen in any of the fractions isolated from Sf9 cells lacking hCTR1 expression plasmids.

The stably expressed hCTR1 is functional, as evident from the kinetics of copper uptake into cells shown in Fig. 2. Specifically, expression of hCTR1 protein is accompanied by a large (about 5-fold) increase in copper uptake (see also TABLE TWO). In this figure we also show the hCTR1-dependent uptake that results from the subtraction of the copper uptake into untransfected cells from the uptake into cells expressing hCTR1. All subsequent data presented on the kinetics of copper uptake into Sf9 cells has been corrected by this subtraction procedure.

Expression and Function of C-terminally Truncated hCTR1—We also investigated the potential functional importance of the C-terminal intracellular tail of hCTR1. We have shown previously, in baculovirus-infected Sf9 cells, that the C terminus can be epitope-tagged without significant disruption of hCTR1 function (20). However, the C terminus was shown to be involved in protein-protein interactions with the cop-
Copper Transport Mediated by hCTR1

per chaperone, Atx1, in vitro (21, 22), suggesting that it may play a role in copper uptake. To test this hypothesis, two C-terminal truncations were generated, CTK178 and CTD184 (Fig. 1A). CTK178 is a construct in which hCTR1 is terminated at Lys178, and CTD184 is a construct in which hCTR1 is terminated at Asp184. The CTD184 construct lacks Cys189, a putative copper-coordinating residue, and CTK178 has most of the C terminus deleted up to a Lys177–Lys178 sequence, which may be important for establishing membrane topology. Both C-terminally truncated constructs are expressed at comparable levels, and both are delivered to the plasma membrane (Fig. 3B, inset).

The functional characterization of these mutants showed that CTK178 and CTD184 were able to mediate copper uptake, although at reduced levels compared with Wt (Fig. 3B and TABLE THREE). The transport capacity of the expressed CTD184 is moderately affected (its transport activity is at about 70% of the Wt hCTR1 levels and the $K_m$ value is very similar to the Wt). However, removal of six additional amino acids significantly reduces the capacity for copper uptake (40% of Wt hCTR1). Most interestingly, the effect of C-terminal deletion on the apparent $K_m$ values is opposite the effect of N-terminal truncations, i.e. the $K_m$ value for copper is increased (TABLE THREE), although the significance of only a 2–3-fold change in the $K_m$ values for copper is difficult to interpret. Taken together, these results suggest that the C terminus does influence, to some extent, the efficiency of copper transport mediated by this protein, perhaps at the copper release step (see “Discussion”).

**Mutants of Met Residues in the Trans-membrane Domains**—The trans-membrane portion of hCTR1 contains four Met residues. The first pair, Met69 and Met81, is located in the first trans-membrane seg-
Copper Transport Mediated by hCTR1

The Synergistic Effect of Cys Substitutions—The above results suggested that neither pair of the intramembrane Met residues was essential for copper transport, although clearly the second pair of methionines (Met\textsuperscript{150} and Met\textsuperscript{154}) was more important for hCTR1 function. Sulfur-containing amino acids are frequently found at copper coordination sites in proteins, and we turned our attention to the two Cys residues Cys\textsuperscript{169} and Cys\textsuperscript{181}. By using baculovirus-mediated infection of SF9 cells, we showed earlier (20) that 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 V\textsubscript{max} values. By utilizing stable cell lines in the present work, we have confirmed that observation (Fig. 4 and TABLE FOUR). We then generated the double C169S/C181S mutant and examined its properties. Unexpectedly, and in marked contrast to single amino acid substitutions, mutations of both cysteines markedly decreased the ability of this construct to transport copper across the membrane. The V\textsubscript{max} value of the double mutant was only 27% of the Wt with little change in the K\textsubscript{m} value for copper (TABLE FOUR). Clearly, simultaneous replacement of the

| Construct | K\textsubscript{m} (μM) | V\textsubscript{max} (pmol of copper/mg of protein/min) |
|-----------|----------------|---------------------------------------------------|
| Wt hCTR1  | 8.9 ± 1.2     | 75.7 ± 4.8                                        |
| SF9       | 5.6 ± 0.8     | 14.7 ± 0.8                                        |
| Wt-Sf9    | 9.2 ± 1.2     | 59.5 ± 2.8                                        |

FIGURE 2. Wt hCTR1 expression in stable SF9 cells. Concentration dependence of copper uptake is shown for SF9 cells expressing either Wt hCTR1 (○) or SF9 cells with no expression of hCTR1 (■). In order to simplify the analysis of hCTR1-dependent transport in SF9 cells by mutant hCTR1 constructs, transport was corrected by subtracting the background uptake levels for SF9 cells lacking hCTR1 protein. The resulting curve for Wt hCTR1 (○) is shown. Inset, Western blot showing fractionated membranes for SF9 cells expressing Wt or without any hCTR1 protein. 20 μg of endoplasmic reticulum (ER), Golgi (G), or plasma membrane (PM) fractions were loaded onto a 12% SDS-polyacrylamide gel. Proteins were visualized using a CT-hCTR1 antibody (1:25,000).

FIGURE 3. hCTR1 truncations. A, concentration dependence of copper uptake by N-terminal truncations in stable SF9 cells expressing N-terminal truncation proteins of hCTR1: Met\textsuperscript{69} (○), MNS3 (□), or MG34 (■). Inset, Western blot of N-terminal truncations expressed in SF9 cells. 20 μg of PM fractions were loaded on 12% SDS-polyacrylamide gels. N-terminal truncation proteins were visualized using a CT-hCTR1 antibody (1:25,000). B, concentration dependence of copper uptake by C-terminal (CT) truncations in stable SF9 cells expressing C-terminal truncation proteins of hCTR1, CT78 (○) or CT184 (■). Inset, Western blot of C-terminal truncations expressed in SF9 cells. 20 μg of PM fractions were loaded on 12% SDS-polyacrylamide gels. C-terminal truncations were identified using an N-terminal hCTR1 antibody (1:10,000).
two cysteines, one in TMS3 and one in the C-terminal tail, resulted in a significant impairment of copper transport. It seems likely that these mutations cause an uncompensated change in local structure with negative consequences to transport (see "Discussion").

The Effect of Tyr Substitutions—Two trans-membrane tyrosine residues (Tyr83 and Tyr147 in hCTR1) are highly conserved among the Ctr family members (Fig. 1B). A third tyrosine residue, Tyr156, is located in the putative extracellular loop between TMS2 and TMS3 and may potentially play a role in copper entry into the trans-membrane pathway. To investigate the role of these residues, Tyr83 and Tyr147 were mutated to either alanine or phenylalanine and stably expressed in Sf9 cells. Analysis of protein expression and copper uptake (Fig. 5) demonstrated that the $K_m$ values of hCTR1 for copper were unaffected in these mutations, although we did observe some effect on the rate of transport.

### Table Four

| Construct | Protein mutant/Wt | $K_m$ (µm) | $V_{max}$ (pmol copper/mg protein/min) |
|-----------|------------------|------------|--------------------------------------|
| M69I      | 0.55             | 4.8 ± 0.4  | 48.7 ± 1.3                           |
| M81I      | 1.25             | 11.7 ± 0.4 | 34.5 ± 0.9                           |
| M150I/M154I | 1.09         | 10.9 ± 2.2 | 17.7 ± 3.9                           |
| C161S/C189S | 1.12          | 11.3 ± 2.8 | 16.3 ± 3.5                           |
| C161S     | 1.02             | 13.2 ± 2.1 | 46.5 ± 2.8                           |
| C189S     | 0.96             | 7.0 ± 2.8  | 51.5 ± 2.2                           |

**FIGURE 4.** Methionine and cysteine substitution mutants of hCTR1. Concentration dependence of copper uptake by methionine mutants and a double cysteine mutant. Stable Sf9 cells expressing either M69I, M81I, M150I/M154I, or C161S/C189S mutant constructs were assayed for $^{64}$Cu uptake. Inset, Western blot of Met mutants expressed in Sf9 cells. 20 µg of plasma membrane fractions were loaded on a 12% gel. hCTR1 mutants were identified using C-terminal hCTR1 antibody (1:25,000).

**FIGURE 5.** Tyrosine substitution mutants of hCTR1. Concentration dependence of copper uptake by representative tyrosine mutants. $^{64}$Cu uptake was measured in Sf9 cells expressing Y83A, Y83F, or Y156A hCTR1 mutants. Inset, Western blot analysis of tyrosine mutants. 20 µg of plasma membrane fractions loaded on a 12% SDS-polyacrylamide gels. Met mutant proteins were identified using a C-terminal hCTR1 antibody (1:25,000).
In each case, the mutant $V_{\text{max}}$ values were 40–50% of Wt for the Tyr to Phe substitutions and 60–70% of Wt for the Tyr to Ala mutants, suggesting that these Tyr residues are unlikely to be directly involved in copper transport.

In contrast, the Y156A mutation had a dramatic effect on copper uptake (TABLE FIVE and Fig. 5). The $V_{\text{max}}$ value of this hCTR1 variant was only 20% of the $V_{\text{max}}$ of the Wt construct, the most significant decrease in the $V_{\text{max}}$ among all single and double mutants examined in this study, and the $K_m$ value for copper was only 2-fold greater than the $K_m$ value of Wt protein.

**Mutations of Charged Residues in the Trans-membrane Domains Result in Gain of Transport Function**—There are three charged residues in the trans-membrane segments of hCTR1, Glu68, Glu84, and His139. The occurrence of charged residues in the trans-membrane segments of cation transport proteins is often associated either with important structural or functional roles, because 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 (Fig. 6, inset). Unlike the mutants described above, these mutant proteins transported copper at higher rates than the Wt hCTR1. E68L and E68Q transported copper at about 1.5-fold higher rate than the Wt hCTR1. The $K_m$ value for the Glu68 mutants is 2–3-fold higher than that of Wt hCTR1 (TABLE SIX). Similarly, the mutants E84L and E84Q also transported copper at higher rates than Wt hCTR1 (1.1- and 1.7-fold, respectively).

The most dramatic and unexpected effect was observed for the H139R mutant, which was designed to alter the size but preserve the structural or functional roles, because 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 (Fig. 6, inset). Unlike the mutants described above, these mutant proteins transported copper at higher rates than the Wt hCTR1. E68L and E68Q transported copper at about 1.5-fold higher rate than the Wt hCTR1. The $K_m$ value for the Glu68 mutants is 2–3-fold higher than that of Wt hCTR1 (TABLE SIX). Similarly, the mutants E84L and E84Q also transported copper at higher rates than Wt hCTR1 (1.1- and 1.7-fold, respectively).

To examine the possible role of His139 in copper transport in more detail, we generated a H139A mutant to replace His with a small and uncharged residue. The Ala mutant showed little effect on $K_m$ for copper (TABLE SIX), suggesting that the effect of Arg substitution on the $K_m$ could be because of the size of this residue. At the same time, the H139A had lower (only 40%) transport activity compared with Wt hCTR1 (TABLE SIX). Thus, the difference in the type of residue at position 139 (Arg compared with Ala) generated in total a 20-fold difference in the rate of transport, with the presence of the (likely) fixed positive charge being highly stimulatory (see also “Discussion”).

**DISCUSSION**

Members of the CTR1 family have no apparent homology to known transporters, and their transport mechanism is currently unknown. In the present work we have carried out mutational and functional analysis of hCTR1 and have made some first steps toward understanding how hCTR1 may mediate copper entry into cells. We have characterized directly and quantitatively the transport activity of various hCTR1 mutants using radioisotopic copper uptake measurements, allowing analysis of hCTR1 function at a greatly improved level of sensitivity and resolution. This was possible because of the utilization of stably expressing insect cells, which provide a permanent and highly reproducible source of the transport protein in uncompromised and directly accessible cell membranes. The unexpected and important finding of this study is that the majority of single residue replacements produce subtle changes in the transport properties of hCTR1. Most of the potential copper-coordinating residues, when deleted by truncation or replaced by substitution, have only a minor effect on the $K_m$ value for transport. Therefore, any binding at these sites does not represent the rate-limiting transport step. Substitution of the intramembrane His139 by Arg, however, causes a large change in both $K_m$ (16-fold) and $V_{\text{max}}$ (8-fold) values and implies that rate-limiting interactions for permeation are likely to take place at this location in the pore.

hCTR1 has only three trans-membrane segments (although the involvement of other portions of the protein into P-loop-like structures cannot be excluded) and forms oligomers with the functional unit being most likely a trimer (18, 23, 24). Neither ATP hydrolysis nor ion gradients seem to be necessary for copper uptake, suggesting that hCTR1 is neither an ion pump nor a secondary transporter. Two fundamentally
different mechanisms for the CTR1-mediated copper transport can be envisioned.

In one mechanism, specific, essential sites accessible to the extracellular membrane surface would bind copper and, following a conformational change, would release copper at the intracellular surface. In a second mechanism, a permeation pathway or pore is formed by the protein, and copper, once having gained access to this pore, passes through the membrane. Such a permanent pore is not a feature of the first mechanism. Access to the pore can be regulated via a gate that "moves" in response to copper loading or in response to some other factor. It seems most likely that a permanent pore structure is formed by the oligomerization of hCTR1. It is possible that some of the changes we have made, for example deletions in the extracellular N-terminal domain (Met69), that result in a dramatic loss of transport function may be the result of destabilization of the functionally essential oligomerization of hCTR1. The apparent absence of essential residues within the intramembrane portion of the protein and the marked stimulation in $V_{\text{max}}$ values caused by the His to Arg substitution at the inner surface suggest a model in which hCTR1 acts through the regulated access to a pore, with rate-limiting interactions occurring close to where copper ions exit from the pore.

Copper Entry into hCTR1——The presence of multiple Met and His residues in the extracellular N-terminal portion of the transporter (the total in a trimer is a remarkable 54!) makes this region an attractive candidate for performing the role of copper "collection" at the mouth of a pore. Previous studies using the yeast complementation assay demonstrated that the second methionine motif (Met40–Met49) was important for copper uptake, whereas the first Met repeat was not (19). Our measurements are consistent with distinct roles of the N-terminal Met residues in the copper transport process.

The deletion of the first 34 residues, which removes all His and half of the Met residues, has no effect on the $V_{\text{max}}$ value of transport and shows only a 2-fold increase in the apparent $K_m$ value for copper. Clearly, this region was not essential for copper entry into the pathway. It was also not essential for the structural integrity of the transporter. However, in

| Construct | Protein mutant/Wt | $K_m$ mutant/Wt | $V_{\text{max}}$ mutant/Wt | $V_{\text{max}}$ (normalized) mutant/Wt |
|-----------|------------------|-----------------|---------------------------|--------------------------------------|
| E68L      | 1.21             | 13.7 ± 2.8      | 1.5                       | 93.4 ± 12.8                          | 1.6 | 1.3 |
| E68Q      | 1.09             | 31.5 ± 3.9      | 3.4                       | 99.5 ± 18.1                          | 1.7 | 1.5 |
| E84L      | 1.23             | 37.1 ± 4.2      | 4.0                       | 82.2 ± 14.3                          | 1.4 | 1.1 |
| E84Q      | 1.06             | 24.0 ± 3.8      | 2.6                       | 105.1 ± 14.1                         | 1.8 | 1.7 |
| H139A     | 1.15             | 11.6 ± 3.1      | 1.3                       | 26.7 ± 5.5                           | 0.4 | 0.4 |
| H139R     | 1.24             | 146.1 ± 4.8     | 15.9                      | 553.2 ± 20.2                         | 9.3 | 7.5 |

FIGURE 7. Model of putative trans-membrane (TM) segments of hCTR1. Putative trans-membrane segments 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 8. Structural model for hCTR1-mediated copper uptake. A two-dimensional diagram of hCTR1 with important amino acid residues labeled is shown. hCTR1 oligomers likely consist of 3 or 4 monomers that form a functional copper transport complex. Trans-membrane segment 2 likely faces the permeation pathway allowing regulation of metal ions as they pass through the membrane. Trans-membrane segments 1 and 3 may provide limited metal-coordinating capacities but may be important for structural interactions within and among monomers.
Copper Transport Mediated by hCTR1

the organism, the delivery of copper to hCTR1 presumably occurs via interactions between extracellular donor protein(s) (the identity of which remains to be resolved) and the extracellular domain of the transporter. Thus, the extracellular domain (with multiple His and Met residues) may play an essential physiological role in accepting copper ions from copper donors. This step is circumvented in the in vitro assay by direct addition of copper ions.

Further deletion of the N-terminal segment (up to 53 amino acid residues) removes all Met and His residues from the extracellular N-terminal domain and has more dramatic consequences for hCTR1 function. The rate of transport decreases significantly (by 70%), with only about a 3-fold change in the $K_m$ value for copper. The marked decrease in the $V_{\text{max}}$ value could be due either to infrequent opening/limited access to the pore or to slower permeation through the pathway. As the permeation pathway is likely to be via a pore through the membrane, any effects of removal of extracellular Met residues are likely to be indirect. The most extensive truncation, Met$^{69}$, removes the entire extracellular N-terminal domain and results in loss of copper transport.

A similar role in facilitating copper entry can be envisioned for Met$^{150}$ and Met$^{154}$. These residues are located at the extracellular portion of TMS2 and are conserved among all Ctrl1 molecules (Fig. 1 and Fig. 7). It is possible that three pairs of Met$^{150}$ and Met$^{154}$ form the entrance into the copper-translocation pathway and facilitate copper entry either through their ability to weakly bind copper and/or by providing an appropriate steric fit for the pore (Fig. 8). It should be noted that the $K_m$ value of the double mutant remains the same as Wt hCTR1, so that it is unlikely that the binding of copper in this location represents a rate-limiting step in the permeation process. Although important, these residues are nevertheless not critical for copper transport. Uptake of copper, although markedly diminished, still occurs at 30% of Wt hCTR1 when both residues are replaced with isoleucines.

These latter data appear at odds with the previous reports (19), which showed that substitutions of Met$^{150}$ and Met$^{154}$ inactivated copper uptake. This apparent discrepancy is most likely due to differences in experimental conditions. Puig et al. (19) measured uptake at an early time point and with a single copper concentration ($2 \mu M$). We have measured copper uptake for a period of 40 min at multiple copper concentrations. If a mutant CTR1 protein transports copper at a very slow rate, comparable with untransfected cells, then a single early measurement may not reveal a small but significant transport capacity.

Tyrosine Mutants in the Trans-membrane Domains—It is known that tyrosine residues are important structurally as hydrogen bond partners in proteins and can also participate in metal ion coordination (25, 26). Most interestingly, the single-site mutation of Tyr$^{156}$ had a dramatic effect on the transport of copper with the Y156A mutant only transporting at a rate of about 20% of Wt hCTR1. This residue is probably located in the short loop between TMS2 and TMS3 and is very close to Met$^{154}$ at the extracellular boundary of the putative transport pore (see Fig. 8). Consequently, it seems likely that Tyr$^{156}$ plays a structural role in helping to place Met$^{154}$ appropriately.

Charged Residues in the Trans-membrane Segments—There are only three charged residues in the transmembrane regions of hCTR1. These residues are located in the first trans-membrane segment (Glu$^{68}$ and Glu$^{86}$) and the second trans-membrane segment (His$^{139}$). Most interestingly, mutations that remove the negative charges on the glutamates increase the rate of transport by hCTR1. Not only is the rate of transport increased, the apparent affinity of these mutations for copper decreases somewhat. It is tempting to speculate that side chain carboxyls may bind to or slow the rate of passage of copper ions through the transport pore as they pass through the membrane. However, the effects are not large, and a more extensive analysis of these sites would be needed to draw definitive conclusions. The same can be said of the M69I substitution in the first trans-membrane segment that results in a slightly increased value (150%) for the $V_{\text{max}}$ for copper uptake.

Copper Exit from the Pore—Very little is known about the immediate fate of copper as it enters the cytoplasm. It has been speculated that copper chaperones accept copper directly from Ctrl1 transporters. Recent in vitro studies demonstrated that the C terminus of yeast Ctrl1 can interact with Atx1 and that copper is transferred between these two proteins (21, 22). Our results show that mutations of C-terminal Cy$^{169}$ or the CT184 deletion have only a slight effect on the rate of transport (10 and 30% decrease, respectively). It should be pointed out that such transfer to chaperones, if it occurs, is unlikely to be rate-limiting for copper uptake. This conclusion can be drawn from our observation that the H139R mutant has a much higher (8-fold) $V_{\text{max}}$ value for transport. It would be unlikely that a mutation within the membrane portion of the transporter could greatly stimulate uptake if the transport process itself was not rate-limiting.

The effects of replacements at position His$^{139}$ are the most dramatic and very interesting. When His$^{139}$ at the cytosolic end of TM2 is mutated to Arg there is a large increase in the $V_{\text{max}}$ value for transport (Ala replacement does not have a similar effect). Such gain-of-function mutations are unusual and therefore are potentially very informative. The specific effect of replacement with Arg could be explained by possible differences in protonation of His (partial) compared with Arg. It is likely that in the Wt hCTR1, all three His$^{139}$ residues (one from each monomer) may not be protonated simultaneously. Mutation to Arg places three positive charges in the pore; this may result in charge repulsions distorting the pore at a critical site, lessening interactions between the pore and the permeating copper ion and increasing the maximal transport rate. The details of the interactions and the involvement of His and Arg residues in the pathway are the subjects of ongoing studies. It is also worth noting that His$^{139}$ is not strictly conserved between yeast and mammalian Ctrl1 proteins (mammalian Ctrl1 proteins have a histidine at this position, and yeast Ctrl proteins do not). However, there is an arginine residue four amino acids away (approximately one turn of an $\alpha$-helix) from this histidine in both yCtrl1 and yCtrl3, which perhaps plays a similar role.

A Working Model for hCTR1—Bringing together the results from this study and others we can begin developing a model for hCTR1 functional architecture (Fig. 8). This model does not yet address the important issue of the interactions of the extramembrane segments of hCTR1 with proteins involved in the delivery to and removal from hCTR1 of copper ions under in vivo conditions. Further experiments are underway to start addressing these questions.

The permeation pathway is formed by the oligomeric association of three hCTR1 molecules. The pore is bounded at the outer membrane surface by a ring of Met residues formed from Met$^{150}$ and Met$^{154}$ in TMS2. Two glycine residues, Gly$^{167}$ and Gly$^{171}$, on the opposed surface of TMS3 have been shown previously to be important for function via their role in helix packing of the trans-membrane domains. The appropriate configuration of the Met residues can be influenced by the nearby Tyr$^{156}$—Cy$^{164}$ is implicated as a potential structurally important residue given our present results with the double mutant C161S/C189S and the results from others (18).

At the inner membrane surface Glu$^{84}$, in TMS1, and His$^{139}$, in TMS2, are close to the exit site from the pore. It is at this latter location that interactions between copper ions and the protein form rate-limiting associations, as evidenced by the dramatic increase in transport rate and $K_m$ value for copper when His is replaced by Arg. We have observed
previously (via proteolytic cleavage studies) that the binding of copper ions (presumably at the extracellular N-terminal domain) causes a conformational change in the intracellular loop between the first and second trans-membrane segments (20). It remains to be shown how such a conformational change contributes to facilitating copper uptake into cells via the proposed hCTR1 pore structure.

Acknowledgments—We thank Dr. Svetlana Lutsenko for critical input and many helpful suggestions. The production of $^{64}$Cu at Washington University School of Medicine was supported by NCI Grant R24 CA86307 from the National Institutes of Health.

REFERENCES
1. Sharp, P. A. (2003) Int. J. Biochem. Cell Biol. 35, 288–291
2. Harris, E. D. (2003) Crit. Rev. Clin. Lab. Sci. 40, 547–586
3. Frieden, E. (1986) Clin. Physiol. Biochem. 4, 11–19
4. El Meskini, R., Culotta, V. C., Mains, R. E., and Eipper, B. A. (2003) J. Biol. Chem. 278, 12278–12284
5. Holm, L., Saraste, M., and Wåstrom, M. (1987) EMBO J. 6, 2819–2823
6. Howard, C. M., Sexton, D. J., and Muzus, B. (1998) Thromb. Res. 91, 113–120
7. Jakub, M., Paret, C., Stucka, R., Horn, N., Müller-Höcker, J., Horvath, R., Trepesch, N., Stecker, G., Freisinger, P., Thirion, C., Müller, J., Lunkwitz, R., Rödel, G., Shoubridge, E. A., and Lochmüller, H. (2001) Hum. Mol. Genet. 10, 3025–3035
8. Medeiros, D. M., and Jennings, D. (2002) J. Bioenerg. Biomembr. 34, 389–395
9. Richter, O. M., and Ludwig, B. (2003) Rev. Physiol. Biochem. Pharmacol. 147, 47–74
10. Petris, M. J., Strausak, D., and Mercer, J. F. (2000) Hum. Mol. Genet. 9, 2845–2851
11. Lutsenko, S., and Petris, M. J. (2003) J. Membr. Biol. 191, 1–12
12. Petris, M. J., and Mercer, J. F. (1999) Hum. Mol. Genet. 8, 2107–2115
13. Roelofsen, H., Wolters, H., and Van Luyn, M. J. (2000) Gastroenterology 119, 782–793
14. Eisses, J. F., Chi, Y., and Kaplan, J. H. (2005) J. Biol. Chem. 280, 9635–9639
15. Tandy, S., Williams, M., Leggett, A., Lopez-Jimenez, M., Redes, M., Ramesh, B., Srai, S. K., and Sharp, P. (2000) J. Biol. Chem. 275, 1023–1029
16. Kuo, Y. M., Zhou, B., Cosco, D., and Gitschier, J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6836–6841
17. Lee, J., Prohaska, J. R., and Tiele, D. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6842–6847
18. Alle, S. G., Eng, E. T., De Feo, C. J., and Unger, V. M. (2004) J. Biol. Chem. 279, 53435–53441
19. Puig, S., Lee, J., Lau, M., and Tiele, D. J. (2002) J. Biol. Chem. 277, 26021–26030
20. Eisses, J. F., and Kaplan, J. H. (2002) J. Biol. Chem. 277, 29162–29171
21. Xiao, Z., and Wedd, A. G. (2002) Chem. Commun. (Camb.) 6, 588–589
22. Xiao, Z., Loughlin, F., George, G. N., Howlett, G. J., and Wedd, A. G. (2004) J. Am. Chem. Soc. 126, 3081–3090
23. Pena, M. M., Puig, S., and Tiele, D. J. (2000) J. Biol. Chem. 275, 33244–33251
24. Lee, J., Peña, M. M. O., Nose, Y., and Tiele, D. J. (2002) J. Biol. Chem. 277, 4380–4387
25. Arresano, F., Banci, L., Bertini, I., Mangani, S., and Thompson, A. R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 3814–3819
26. Whittaker, I. W. (2005) Arch. Biochem. Biophys. 433, 227–239