Molecular Characterization of hCTR1, the Human Copper Uptake Protein*

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We have expressed hCTR1, the human copper transporter, in Sf9 cells using a baculovirus-mediated expression system, and we observed greatly enhanced copper uptake. Western blots showed that the protein is delivered to the plasma membrane, where it mediates saturable copper uptake with a $K_m$ of $\approx 3.5 \mu M$. We also expressed functional transporters where the $N$-linked glycosylation sites were substituted, and we provided evidence for the extracellular location of the amino terminus. Accessibility of amino-terminal FLAG epitope to antibody prior to permeabilization and of carboxy-terminal FLAG only after permeabilization confirmed the extracellular location of the amino terminus and established the intracellular location of the carboxyl terminus. Tryptic digestion of hCTR1 occurred within the cytoplasmic loop and generated a 10-Da carboxyl-terminal peptide; cleavage was prevented by the presence of copper. hCTR1 mutants where Cys-161 and Cys-189, the two native cysteines, were replaced with serines also mediated copper uptake, indicating that neither cysteine residue was essential for transport. However, the mutants provided evidence that these residues may stabilize hCTR1 oligomerization. Western blots of hCTR1 in Sf9 cells showed expression levels 100-fold higher than in mammalian (HepG2) cells. The high level of functional expression and the low level of endogenous copper uptake will enable future structure-function analysis of this important protein.

It has been estimated that about one-third of all known proteins contain metal cofactors, and the majority of these function as essential metalloenzymes. Copper is an essential micronutrient in humans, and it is required for cellular respiration, iron homeostasis, pigment formation, neurotransmitter biosynthesis, peptide biogenesis, connective tissue production, and antioxidant defense (1). Recent studies (2–8) in microorganisms and the identification and characterization of the molecular basis of several genetic diseases of copper metabolism in humans have provided a partial list of the components involved in regulating and controlling intracellular copper metabolism. The transport of copper into and out of cells has also received increased attention. Mutations in either of two human genes encoding copper-transporting P-type ATPases that are localized in the secretory pathway result in Menke’s disease (ATP7A) and Wilson’s disease (ATP7B), which cause intestinal copper absorption defects or maldistribution of hepatic copper, respectively, (3, 4). The two P-type ATPases are clearly essential for the controlled removal of copper from cells (9–13). The importance of these proteins was established in 1993; however, at that time the mechanism of copper uptake into mammalian cells was entirely unknown.

An important series of studies in the yeast Saccharomyces cerevisiae, first appearing in 1994, provided the basis for our current ideas about the major copper entry mechanisms (14, 15). It was shown that a gene, CTR1, encoded a multispanning plasma membrane protein that was specifically required for high affinity copper transport into yeast. The CTR1 gene product is a protein composed of 406 amino acid residues. A second high affinity copper uptake protein, encoded by the CTR3 gene, was subsequently identified. The two gene products are functionally redundant and both contain three putative transmembrane segments; however, they are structurally quite different (16). CTR3 is composed of 241 amino acid residues, and it lacks the MXMXM motifs that are abundant in CTR1. CTR3 is relatively rich in Cys residues, containing 11 Cys residues in its sequence, although apparently only 4 of these residues may be important for function (16). CTR1 is highly glycosylated (14) and has eight repeats of the MXMXM motifs. It was speculated that the glycosylation had some functional significance (14). The Met motifs, which are regarded as potential metal-binding sites, are located in the amino-terminal tail that is thought to be in the predicted extracellular domain. The presence of these multiple sulfur-containing amino acid residues suggested a possible role in copper coordination. It should be borne in mind that the hydrophathy analysis of these transporters suggests three transmembrane segments (see Fig. 1) and thus the amino and carboxyl termini are on opposite sides of the membrane. The reason that the amino terminus was placed on the outside in the initial models was because the Met domains were in the amino terminus, and since the proteins were copper uptake systems, copper binding was assumed to be the first step in the process. Obviously two alternatives exist, with the amino terminus at the extracellular surface and the carboxyl terminus in the cytosol, as assumed, or vice versa.

In 1997 Zhou and Gitschier (17) identified the first human gene for copper uptake. This protein, hCTR1, was identified by complementation of a CTR1 growth defect in yeast on non-fermentable media and also rescued iron transport and copper/zinc superoxide dismutase defects. The gene was proposed to encode a high affinity copper uptake process and was found to be 29% identical at the primary structure level with Ctr1 from yeast. The human protein was, however, substantially smaller than the yeast protein, being composed of 190 amino acids as compared with 406 amino acids (see Fig. 1). hCTR1 is on the one hand a smaller protein, like yCtr3 (241 residues) but has

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Met motifs like 

The amino-terminal domain of hCTR1 has been described as having two Met motifs; however, the first of these is MXXM, rather than the usual MXXMX, and the second is unusually methionine-rich in having the sequence MMMXM. hCTR1 also has a methionine-rich sequence at the extracellular boundary of the second putative transmembrane segment that may be important for function. hCTR1, in contrast to CTR3, is a very Cys-poor protein having only two Cys residues, Cys-161 and Cys-189, in its sequence. The murine homolog of hCTR1, termed mCTR1, was isolated by Lee et al. (18) 3 years later and was shown to have 92% protein sequence identity with hCTR1. Mouse CTR1 (mCTR1) in fact has only 4 residues that differ from hCTR1 beyond the first 37 residues. Mouse CTR1 complemented yeast strains that were defective in high affinity copper uptake. The mammalian CTR1-RNA was expressed in all tissues examined, with higher levels in kidney and liver and lower levels in brain and spleen (18). Recent studies (19, 20) have established that expression of mammalian CTR1 is essential for embryonic development. It was shown that homozygous knock-outs resulted in embryonic lethality, whereas heterozygous animals exhibited tissue-specific defects in copper accumulation and in the activities of copper-dependent enzymes. In a publication that appeared during the preparation of this article (21), an initial characterization of hCTR1 in mammalian cells was reported. The overexpression of hCTR1 in HEK 293 cells was described and evaluated by measuring $^{64}$Cu uptake. These authors showed time-dependent, saturable copper uptake. By utilizing mutagenic analysis of expressed hCTR1 molecules, we show that neither of the two Cys residues, Cys-161 or Cys-189, play a role in copper uptake and that glycosylation of hCTR1 is not required for function. By combining these results with experiments on proteolytic susceptibility and antibody accessibility, we provide strong evidence for a three-transmembrane segment model with an extracellular amino terminus and an intracellular carboxyl terminus. The functional heterologous expression of hCTR1 in insect cells will enable a detailed investigation of the mechanism of this important transporter.

**EXPERIMENTAL PROCEDURES**

**Cloning of hCTR1**—The cDNA for hCTR1 was obtained from the laboratory of Dr. Jane Gitschier, University of California, San Francisco. hCTR1 was PCR-cloned into pFastBacDual vector (pFBD; Invitrogen) as an EcoRI-HindIII fragment. Additionally, hCTR1 was PCR-amplified to insert a FLAG epitope (NH$_2$-DYKDDDDK-COOH) at the amino terminus of hCTR1 as well as a construct containing a FLAG epitope at the carboxyl terminus of hCTR1. Mutagenic primers were designed to introduce single amino acid substitutions at amino acids N15Q, N112Q, C161S, and C189S (Table I). Mutant cDNAs of hCTR1 were cloned into pFBD vector (Invitrogen) as EcoRI-HindIII fragments. The cloned fragments were used to generate recombinant baculovirus following the manufacturer’s protocols (Invitrogen).

**Transfection and Expression of hCTR1 in Insect Cells**—Briefly, cloned mutant cDNA were allowed to transpose into recombinant bacmids within DH10 Bac cells (Invitrogen). Colonies containing hCTR1 mutant bacmids were then used to produce virus particles by transfixing Sf9 insect cells with the recombinant back DNA. The resulting virus stocks were amplified twice and then used to express protein using Sf9 cells (22). Sf9 cells were infected at a cell density of 1.0 $\times$ 10$^8$ cells/ml in spinner flasks. Cells were collected and disrupted via Dounce homogenization to produce unfraccionated or fractionated membranes (22, 23) containing overexpressed hCTR1, followed by Western analysis.
Characterization of hCTR1

Western analysis—Western analysis was performed using 50 μg of membranes loaded on a 12% Laemmli gel. β-Mercaptoethanol (0.2% v/v) was added to samples 15 min prior to electrophoresis at room temperature unless indicated. The gel was electroblotted to nitrocellulose membrane in 10 mM CAPS, pH 11, and blocked using phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.4) containing 5% milk. After blocking, the membranes were incubated with a rabbit anti-carboxyl-terminal hCTR1 antibody (NH₂-WKKAVVVDITEHCH-COOH; 1:5000 dilution), and secondary detection was carried out using a goat anti-rabbit antibody (1:5000).

Copper Transport by hCTR1—Copper uptake experiments were performed following previously published protocols (24) for rubidium uptake. Briefly, 12-well plates were seeded with Sf9 cells (1.0 × 10⁶ cells/well). Virus was applied to each well of attached cells and allowed to incubate at 27 °C for 72 h. Virus containing either an NT-FLAG or CT-FLAG construct was labeled using a mouse monoclonal anti-FLAG antibody (Anti-FLAG M2 monoclonal antibody, Sigma). Briefly, Sf9 cells were infected with baculovirus particles containing either an NT-FLAG or CT-FLAG construct and allowed to incubate for 72 h. The cells were pelleted at 500 g for 1 min and washed 3 times with ice-cold incubation buffer containing 10 mM EDTA. Cells were lysed using 0.1 N NaOH, and aliquots were counted using a γ-counter (Packard Instruments, Cobra II) and also used to determine protein concentrations. Data were fit using the Michaelis-Menten equation. The levels of expressed hCTR1 mutant protein were monitored by Western analysis using a carboxyl-terminal hCTR1 antibody as described above. PNGase F treatment was performed on unfractionated membranes isolated from Sf9 cells expressing either N15Q hCTR1 or N112Q hCTR1. Briefly, 30 μg of membranes from each mutant sample were treated with 0.1% SDS, 50 mM β-mercaptoethanol for 15 min at room temperature. Nonidet P-40 was added to a final concentration of 0.75%. PNGase F (2.5 milliunits, Glyco, Novato, CA) was added, and the mixture was incubated at 37 °C for 8 h. Samples were run on a 12% Laemmli gel and analyzed by Western blot analysis. Antibodies raised in rabbits against the carboxyl terminus of hCTR1 were used in Western analysis following the protocol described above.

FLAG Antibody Labeling of Amino-terminal and Carboxyl-terminal Tagged hCTR1—Amino-terminal and carboxyl-terminal FLAG epitope-tagged hCTR1 (NT-FLAG or CT-FLAG) expressed in Sf9 cells were used to probe the topology of hCTR1. Intact Sf9 cells were surface-labeled using a mouse monoclonal anti-FLAG antibody (Anti-FLAG M2 monoclonal antibody, Sigma). Briefly, Sf9 cells were infected with baculovirus particles containing either an NT-FLAG or CT-FLAG construct and allowed to incubate for 72 h. The cells were pelleted at 500 × g and resuspended in phosphate-buffered saline. One-half of the cells was used to prepare membranes for antibody labeling (membranes (M)), and the other half was used to label whole cells with antibody (intact cells (IC)). Antibody labeling was carried out for 2 h at 4 °C for both samples (M and IC, NT-FLAG and CT-FLAG hCTR1). The cell suspension was washed 4 times with phosphate-buffered saline containing 1 mg/ml bovine serum albumin for 15 min at 4 °C. The IC samples were then used to prepare total membranes. Unfractionated membranes (500 μg) were solubilized in incubation buffer (150 mM NaCl, 10 mM KCl, 2.5 mM MgCl₂, 5 mM Hepes, pH 7.4) containing 2% n-dodecyl-β-D-maltoside (DDM buffer; Calbiochem). Following solubilization, the samples were diluted to 0.2% DDM buffer, and protein G-Sepharose (Amersham Biosciences) was added for an overnight incubation at 4 °C. The protein G-Sepharose was pelleted at 500 × g, followed by three 1-ml washes with 0.2% DDM buffer. Protein G-Sepharose was resuspended in 2× sample buffer (equal volumes of 8 M urea, 10% SDS, and 125 mM Tris buffer, pH 6.8, no β-mercaptoethanol added) and run on a 12% Laemmli

| Primer          | Primer orientation | Nucleotide Sequence                  |
|-----------------|--------------------|--------------------------------------|
| N-terminal Flag hCTR1 | Forward | 5'-gaattcatggactacagaagcgactgagaatggatcactc-3' |
|                 | Reverse           | 5'-aagcttacaaactcccaacgtc-3'       |
| C-terminal Flag hCTR1 | Forward | 5'-gctgtggactccccagcgacagagactccc-3'          |
|                 | Reverse           | 5'-gcagagcagctcagacgtc-3'          |
| hCTR1           | Forward           | 5'-gcatccagctcactgccgtgctg-3' |
|                 | Reverse           | 5'-gctctctgctgctgctg-3' |
| N15Q            | Forward           | 5'-gccttcctgactgctgctgctg-3'       |
|                 | Reverse           | 5'-gcagagcagctcagacgtc-3'          |
| N112Q           | Forward           | 5'-gctctctgctgctgctgctg-3'       |
|                 | Reverse           | 5'-gcagagcagctcagacgtc-3'          |
| C161S           | Forward           | 5'-gctctctgctgctgctgctg-3'       |
|                 | Reverse           | 5'-gcagagcagctcagacgtc-3'          |
| C189S           | Forward           | 5'-gctctctgctgctgctgctg-3'       |
|                 | Reverse           | 5'-gcagagcagctcagacgtc-3'          |

The abbreviations used are: CAPS, 3-(cyclohexylamino)propanesulfonic acid; PNGase F, peptide N-glycosidase F; CT-FLAG, carboxyl-terminal FLAG; NT-FLAG, amino-terminal FLAG; IC, intact cells; M, membranes.
gel, followed by Western analysis (see above) using a rabbit anti-FLAG antibody (1:5000 dilution; ABCAM, Cambridge, UK).

Trypsin Digestion of hCTR1—Trypsin digestion of C189S hCTR1 was carried out on unfractionated membranes (TM) from Sf9 infected insect cells. TM samples (100 μg) of C189S were incubated with 40 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin in incubation buffer for 1 h at 27°C. 80 μg of soybean trypsin inhibitor was added for 30 min, and then samples were washed twice with incubation buffer to remove any remaining trypsin. TM samples (50 μg) were loaded on a 12% Laemmli gel for subsequent Western analysis using an anti-carboxyl-terminal hCTR1 antibody for detection.

RESULTS

Functional Expression of hCTR1—We have recently optimized the baculovirus infection of Sf9 cells for the heterologous expression of the renal Na⁺,K⁺-ATPase-ATPase (22–24) and for ATP7B, the Wilson’s disease protein (25). In the present work we have infected Sf9 cells with a baculovirus construct containing the cDNA for hCTR1 (provided by Dr. Jane Gitschier, University of California, San Francisco), and after 3 days of infection, the cell membranes were separated using sucrose gradient sedimentation to yield endoplasmic reticulum, Golgi apparatus, and plasma membrane fractions (22). Western blot analysis using an antibody raised against a peptide derived from a sequence in the carboxyl terminus of the transporter (see “Experimental Procedures”) showed that the infected cells synthesized hCTR1, and following synthesis, hCTR1 was delivered to the plasma membrane (Fig. 2A). Fig. 2A shows the major bands of hCTR1, in each membrane fraction, which corresponds to monomeric protein. However, multimerization (i.e. bands at higher mass) was seen in all fractions and is discussed further below (see “The Role of the Cysteine Residues in hCTR1” and Fig. 6). When we optimized this expression system for the sodium pump, we developed an ⁶⁷Rb isotopic uptake assay for the transport properties of the plasma membrane-incorporated expressed protein (24). In the present studies, we modified this protocol to measure the rate of copper uptake into uninfected Sf9 cells and cells infected with baculovirus particles that contained hCTR1 cDNA. The results of such experiments are shown in Fig. 3. It was clear that infected cells showed a greatly enhanced rate of copper uptake compared with uninfected cells (Fig. 3A). If the studies were carried out at varied levels of extracellular copper, two points were immediately apparent. First, infected cells expressing hCTR1 showed an enhanced copper uptake rate that saturates with increasing copper concentrations, and second, uninfected cells showed only very low rates of uptake (Fig. 3B). In
FIG. 3. Copper transport by cells expressing hCTR1. 12-Well plates were seeded with Sf9 cells (1.0 × 10^6 cells/well). 72 h post-infection, cell media were replaced with transport buffer (150 mM NaCl, 2.5 mM MgCl₂, 25 mM HEPES, pH 7.4) and incubated at 27 °C for 30 min. Copper transport was carried out using either ^{64}Cu or ^{67}Cu at specified CuCl₂ concentrations. A, time course experiments were performed at 10 μM CuCl₂. Shown here is an experiment using uninfected cells (solid squares), Sf9 cells expressing the β-subunit of Na⁺/K⁺-ATPase (open diamonds), or Sf9 cells expressing hCTR1 (open squares). hCTR1 mediates at least a 3–4-fold increase of copper uptake into Sf9 cells. B, saturable copper uptake by Sf9 cells expressing hCTR1 is shown for a typical experiment. Different CuCl₂ concentrations were used to measure copper uptake by uninfected cells (solid squares) or cells expressing hCTR1 (solid circles, Kₘ = 3.2 μM). Cells were lysed using 0.1 n NAOH, and aliquots were counted using a γ-counter (Packard Instruments Cobra II) and also used to determine protein concentrations. Expression levels of the hCTR1 mutants were monitored by Western analysis.

fact, in the hCTR1-expressing cells, the maximal rate of copper uptake was some 20–30-fold higher than in the uninfected cells. The copper concentration at half-maximal uptake rates was about 3–4 μM. This value agreed quite well with that recently reported (21) in studies where hCTR1 was expressed in HEK 293 cells.

Because we expressed hCTR1 in Sf9 cells in the hope that its expression would be high so that we could carry out a characterization of its properties, we thought it would be interesting to compare the expression level we obtained in insect cells to that endogenously expressed in a mammalian cell line. In Fig. 2B, we show a comparison of the Western blots of heterologously expressed hCTR1 in Sf9 cell membranes and the endogenously expressed hCTR1 in HepG2 cells. Clearly expression in the insect cell system was greater, and we estimated that the expression in the insect cells was 100-fold higher than in the HepG2 cells. In Fig. 2B, it is apparent that the mobility of hCTR1 in HepG2 cells (35 kDa) differs from that of monomeric hCTR1 (28 kDa) expressed in Sf9 cells. We believe that this was due to the more extensive glycosylation of membrane proteins in mammalian cells compared with insect cells. The band at 46 kDa, in the insect cells, was due to stable oligomerization of wild type protein (see below and Fig. 6 for “Discussion”). We have not observed multimerization of endogenous hCTR1 in HepG2 cells. The significance of this difference is currently under investigation.

Membrane Topology of hCTR1—Hydropathy analysis of the sequence of hCTR1 strongly suggested the presence of three transmembrane segments (see Fig. 1). It has been assumed that the amino terminus is extracellular and the carboxyl terminus is located in the cytosol. We noticed that the hydropathy analysis placed the two consensus sequences for N-linked glycosylation sites, Asn-15 and Asn-112, on opposite sides of the first putative transmembrane segment (Fig. 1B). There are two possible membrane topologies if hCTR1 has three transmembrane segments (see Fig. 4A). We then engineered and expressed two substitution mutants N15Q and N112Q of hCTR1, reasoning that if the hydropathy analysis established the correct number of transmembrane segments with the amino terminus at the extracellular surface, then Asn-15 would be exposed and glycosylated, whereas N112Q would not be glycosylated as it would be in the cytoplasm. If were going to use these substitution mutants to address the membrane orientation of hCTR1, it was first necessary to establish that such substitution mutants were functional. In Fig. 4B, we show that the apparent affinity for copper uptake is largely unaffected by these substitutions. These data also indicate that the absence or presence of glycosylation has no obvious effect on the transport functions of hCTR1. The data, shown in Fig. 4C, support our speculations on the probable membrane orientation and glycosylation status of hCTR1. Expression of the N15Q hCTR1 produced a protein that had an apparent mass of 23 kDa, which was unaltered by treatment with PNGase F. Expression of the N112Q mutant produced a protein of 28 kDa (like hCTR1, see Fig. 2), which was lowered to 23 kDa on treatment with PNGase F. This established N112Q was glycosylated and that Asn-15 was the site of attachment of N-linked glycosylation. Asn-15 was thus exposed to the extracellular medium, supporting the model that places Asn-112 in the cytoplasm (see Fig. 4A).

By having established that the amino terminus of hCTR1 was exposed to the extracellular medium, the presence of three putative transmembrane segments predicted an intracellular carboxyl terminus. In order to provide experimental support for this hypothesis, we carried out labeling experiments on Sf9 cells expressing hCTR1 molecules bearing epitope tags at each terminus. It was first necessary to establish that these modifications did not produce inappropriately folded transporters. In Table II, we show the results of copper uptake assays carried out on Sf9 cells expressing amino-terminal and carboxyl-terminal FLAG-tagged hCTR1 proteins. Clearly both constructs are able to mediate copper uptake with Kₘ values that are unchanged by the substitutions. Thus it is reasonable to assume that these tagged molecules are appropriately folded. In Fig. 5, we show that the amino-terminal FLAG-epitope is detected equally well in whole cells or membranes, i.e. whether or not
the Sf9 cells are permeabilized prior to FLAG antibody addition. In contrast, the FLAG epitope was not detected at the carboxyl terminus of hCTR1 in whole cells, only if the cell membrane was disrupted by homogenization prior to antibody addition. These results confirmed the extracellular location of the amino terminus of hCTR1 that was strongly suggested by our glycosylation mutants described above, and established that the carboxyl terminus of hCTR1 was in the cytosol as
TABLE II

| hCTR1 construct     | Apparent $K_m$ | No. experiments |
|---------------------|----------------|-----------------|
| hCtri               | 3.62 ± 0.85    | 4               |
| N15Q                | 5.90 ± 1.52    | 4               |
| N112Q               | 3.24 ± 0.62    | 5               |
| C161S               | 8.19 ± 1.45    | 4               |
| C188S               | 11.89 ± 0.70   | 4               |
| NT-FLAG             | 5.19 ± 1.01    | 5               |
| CT-FLAG             | 10.08 ± 2.99   | 3               |

FIG. 5. Anti-FLAG antibody labeling of epitope-tagged hCTR1. Amino-terminal and carboxyl-terminal epitope-tagged hCTR1 expressed in Sf9 cells were used to probe the amino- (NT) and carboxyl (CT)-terminal topology of hCTR1. Intact Sf9 cells were either surface-labeled (IC) using a mouse monoclonal anti-FLAG antibody or used to prepare unfraccionated membranes (M) for antibody labeling. Antibody labeling was carried out for 2 h at 4°C for both samples. The intact cell samples were then used to prepare membranes. 500 µg of total membrane preparations were solubilized in 2% DDM buffer. The solubilized membranes were then used in an immunoprecipitation as described under "Experimental Procedures."

required if indeed there are an uneven number of transmembrane segments, as predicted by the hydropathy analysis.

The Role of the Cysteine Residues in hCTR1—hCTR1 has only two cysteine residues. In many copper-binding proteins coordination of the metal ion by sulfur centers are important. We explored the role of the cysteine residues in hCTR1 by replacing each of them with serines. In each case function was unaltered, and $K_m$ values were obtained that are close to the values of unsubstituted hCTR1 (see Table II), suggesting that metal coordination in hCTR1 did not involve these cysteine sulfur centers.

Western analysis of hCTR1 cysteine mutants revealed that these residues might be involved in multimer stabilization. Studies with yCtri and hCTR1 have demonstrated the ability of these molecules to form multimers in the presence of a cross-linker (16). Similarly, wild type hCTR1, N15Q, N112Q, and hCTR1 molecules containing an amino-terminal FLAG epitope exhibited some retention of dimer formation even after migration through an SDS-PAGE gel in the presence of 6% SDS (see Fig. 6). The cysteine substitution mutants did not show this stability; C188S in particular showed only mobilities correlated with the monomeric species when probed with hCTR1 carboxyl-terminal antibody (Fig. 6). The N-terminal FLAG construct showed predominantly the dimeric species (Fig. 6), whereas the carboxyl-terminal FLAG construct showed less dimer stabilization than other hCTR1 molecules (data not shown). When samples of hCTR1 were treated with β-mercaptoethanol (65°C), the presence of dimers was reduced significantly (Fig. 6). β-Mercaptoethanol treatment also reduced the presence of dimers for wild type as well as all other mutant forms of hCTR1 (data not shown).

**FIG. 6. The role of cysteine residues in the formation of hCTR1 multimers.** Amino-terminal FLAG epitope-tagged hCTR1 and C188S hCTR1 (50 µg) were left untreated or treated with 0.2% β-mercaptoethanol (β-ME), 65°C, for 15 min in SDS sample buffer and then run on 12% Laemmli gels to investigate the role of cysteine residues in formation of multimers. Following Western analysis with anti-hCTR1 antibody, the presence of multimers in lanes containing NT-FLAG hCTR1 is contrasted with the lack of multimers in C188S lanes. Addition of β-mercaptoethanol reduces the amount of multimerization in NT-FLAG sample while leaving C188S sample unaffected.

Conformational Changes in the M1M2 Loop—Hydropathy analysis of hCTR1 suggests that the first transmembrane segment begins close to residues 59–63. This places the two Met domains of the amino terminus in the extracellular medium, appropriately placed for copper coordination. Furthermore, this would place a single potential tryptic cleavage site (at Lys-52) in the extracellular domain and several potential cleavage sites (Lys-87, Arg-90, Arg-95, Arg-102, and Lys-121) in the first intracellular loop between transmembrane segments 1 and 2. In order to establish whether or not this was correct, we examined the effects of trypsin treatment on membranes isolated from Sf9 cells that expressed hCTR1. In Fig. 7, we show the results of such experiments. Trypsin treatment of membranes produces a stable 10-kDa fragment that is visible after Western analysis using our carboxyl-terminal antibody. The size of this fragment is consistent with a cleavage at one of the more amino-terminal arginines or lysines in the cytoplasmic loop between transmembrane segments 1 and 2 (see "Discussion"). In order to distinguish cleavage at Lys-52 from intracellular cleavage sites, we compared the mobilities of the proteolytic 10-kDa fragment with that of an expressed truncation of hCTR1, beginning at Val-62. This polypeptide has an apparent mobility corresponding to 16 kDa (see Fig. 7B). This is clearly significantly greater than the tryptic cleavage product of hCTR1. The presence of copper during treatment with trypsin resulted in protection against proteolytic digestion (see Fig. 7).

DISCUSSION

In the present work we have characterized the properties of hCTR1, the human copper uptake protein, following its expression in Sf9 cells. We provide evidence in support of a three transmembrane segment topology, with an extracellular amino terminus and an intracellular carboxyl terminus. The protein is expressed in a functional form in this system, and we have...
In order to distinguish between the two putative membrane orientations of hCTR1, we constructed substitution mutants at Asn-15 and Asn-112. Two different membrane orientations are predicted by hydropathy analysis of the hCTR1 amino acid sequence: one of the two consensus glycosylation sites would be extracellular (Asn-15), and the other would be intracellular (Asn-112) or vice versa (Fig. 4A). In both models, the first putative transmembrane segment separates Asn-15 and Asn-112. Only extracellular sites of plasma membrane proteins become glycosylated at NXS and NXT consensus sequences in the lumen of the endoplasmic reticulum. Our data show that the correct orientation for hCTR1 is the first of the two models shown in Fig. 4A. Expression of the substitution mutants reveals that the N15Q mutant has a lower molecular weight than the other substituted mutant (N112Q) and that the apparent mass of N15Q (23 kDa) is unaffected by treatment with PNGase F, which removes glycosylation moieties. The N112Q mutant, on the other hand, has the same molecular weight as the wild type protein and our cysteine-substituted mutants, and like these forms of the protein, its molecular mass is reduced by the treatment with PNGase F (Fig. 4C). Thus our data clearly support the first orientation shown in Fig. 4A depicts the membrane orientation of hCTR1.

Our transport data on the N15Q mutant also eliminate any essential involvement of protein glycosylation for the copper transport process. It was earlier observed that YCTR1 was highly glycosylated, and it was speculated that this glycosylation might play a role in transport (14). This does not seem to be the case for hCTR1 in insect cells.

To characterize further this suggested topology, we used hCTR1 molecules that contained the FLAG epitope fused to either the amino or carboxyl terminus of hCTR1. By probing cells that were expressing epitope-tagged hCTR1 with a FLAG antibody before and after disruption, we could ascertain which end of the molecule was extracellular and which was intracellular. As seen in Fig. 5, the FLAG antibody detects the amino-terminal epitope-tagged hCTR1 equally well whether labeled in whole cells or permeabilized cells. This suggests that the amino terminus is located outside the cell in agreement with our glycosylation data. In addition, the carboxyl-terminal epitope-tagged hCTR1 was labeled much more extensively in the permeabilized cells as compared with the intact cells. This is the result we would expect if the FLAG epitope were located inside the cell. These data supply strong evidence that the first topological model in Fig. 4A is correct.

hCTR1 is susceptible to trypsin treatment, containing at least one cleavage site within the cytoplasmic loop between transmembrane segments 1 and 2. This result lends support to the topology that has been suggested by the other results in this paper. We have demonstrated that the amino terminus of hCTR1 is located extracellular and its carboxyl terminus is intracellular. The loop between the first transmembrane and the second transmembrane segment is intracellular and has several putative trypsic cleavage sites. Trypsin treatment of hCTR1 in the absence of copper produces a stable 10-kDa fragment. The trypsin cleavage site is most likely after the first transmembrane domain for the following two reasons. The 10-kDa fragment is a carboxyl-terminal fragment as our carboxyl-terminal fragment clearly agrees most closely with cleavage at these sites.

Fig. 7. Trypsin digestion of hCTR1. A, hCTR1 membranes (C189S, 50 μg) were digested with trypsin for 60 min at room temperature in the presence and absence of 10 μM CuCl2. The digestion was stopped by the addition of soybean trypsin inhibitor and subsequent addition of sample buffer (6% SDS). Samples were run on 12% Laemmli gels and blotted to nitrocellulose membranes for Western analysis with an antibody to hCTR1. B, to localize the potential trypsic cleavage site that produces the stable 10-kDa fragment seen in lane 2, we expressed an epitope-tagged amino-terminal truncation hCTR1 molecule (Val-62–His-190). It bears an amino-terminal tag (MASYSH-PQFERGKETVAVPNS) that can be used for detection. A 12% Laemmli gel was run with untreated C189S hCTR1 (lane 1), trypsin-treated C189S hCTR1 (lane 2), and the amino-terminal truncated hCTR1 (lane 3). The blot was analyzed using a carboxyl-terminal hCTR1 antibody.

Table 1. Characterization of hCTR1

| Orientation | Membrane Topology of hCTR1—The amino acid sequence of hCTR1 when examined by hydropathy analysis gives three clear transmembrane segments, which thus predicts either of the two topologies in Fig. 4A. The present work was undertaken to establish which of these orientations was correct, in a system where the hCTR1 molecule was functional. Recently, we have optimized baculovirus infection of insect cells for the characterization of structure-function relations in the renal Na+K+-ATPase-ATPase (22–24) as well as ATP7B, the Wilson’s disease protein (25). In the present work, we show that this system is also appropriate for the functional expression of hCTR1 and that hCTR1 expression in insect cells mediates copper uptake with a $K_m$ of $-3.5 \pm 1.3 \, \mu M$, which is close to the $K_m$ values recently reported by Lee et al. (21).

In order to distinguish between the two putative membrane
an apparent mobility of 16 kDa (Fig. 7B). Thus the cleavage site obtained with trypsin produces a considerably smaller fragment than the predicted cleavage at Lys-52 and is probably just after the peptide emerges from the membrane in the cytoplasmic loop between transmembrane segments 1 and 2. In the presence of 10 μM CuCl₂, the full-length hCTR1 molecule is protected from digestion suggesting that a conformational change in the cytoplasmic loop between transmembrane segments 1 and 2 has occurred following substrate binding. We are currently investigating the precise site(s) of cleavage, as this conformational change will be an important aspect of ligand-induced structural changes accompanying the transport process.

The membrane orientation we have determined has been assumed in previous work on yeast (14–16) and mammalian copper uptake proteins (18, 21). Presumably this is because the proteins mediate copper uptake, and the presence of the MXXMXXM motifs (Met motifs) in the amino terminus suggests that this may be the site of initial copper binding by the protein. The present work puts this topology proposal on firm experimental ground.

**The Role of Cysteine Residues in hCTR1**—In the other closely related yeast proteins, CTR1 and CTR3 (16), there are numerous cysteine residues that may play a role in copper transport. hCTR1 has only two cysteine residues, and our work shows that neither is essential for function. The Km value for each of the cysteine-substituted mutants is similar to wild type hCTR1 in our system (see Table II). Neither of the two cysteines is conserved in other non-mammalian CTR1 proteins. Therefore, our work and the lack of conservation of these residues in closely related copper transport proteins suggests strongly that copper-protein interactions during transport must occur at other candidate copper-coordinating residues, such as methionine (in Met motifs) or histidine.

Additionally, our cysteine substitution mutants do provide some evidence that the oligomerization of hCTR1 described previously (16, 21) may be mediated through interactions at its carboxyl-terminal end. Because hCTR1 has only 190 amino acids and contains only three membrane segments, it has been suggested that the functional form of hCTR1 is probably a multimer. Pena et al. (16) have shown that yCtr3 and more recently hCTR1 does indeed form multimers (21). In the presence of cross-linker, stable multimers of hCTR1 can be obtained. The structure and interactions of these multimers is unknown. We provide evidence that suggests the carboxyl terminus of hCTR1 may play a role in the formation and stabilization of functional hCTR1 complexes. hCTR1 molecules expressed in insect cells form stable oligomers even in the presence of SDS in Laemmli gels (see Fig. 6). Treatment of NT-FLAG-tagged hCTR1 with β-mercaptoethanol dramatically reduces the amount of oligomerization seen in SDS gels (see Fig. 6), implying that S–S bridge formation may be involved in stabilizing these multimers. In support of this, the C189S mutant shows only monomeric species in SDS gels (see Fig. 6). However, since C189S is functional in copper transport (see Table II), the formation of such putative S–S bridges cannot be essential. In keeping with the idea that the carboxyl-terminal segment is involved in monomer-monomer interactions and stabilization of multimers, our CT-FLAG construct also shows predominantly monomeric species in SDS gels (data not shown), as if the epitope interferes with such interactions. Further experiments are underway to elucidate a functional role (if any) for multimerization. Currently, it appears that detergent stability of hCTR1 complexes may be facilitated by S–S bond formation, but such bonding is not essential for function. If functional hCTR1, by necessity, forms multimers, other interactions must occur to facilitate their formation in the native state.

The agreement in the apparent affinities of hCTR1 for copper, following expression in mammalian cells and insect cells, coupled with the very low endogenous copper uptake activity of SF9 cells, indicates that the baculovirus-mediated expression in insect cells will be highly appropriate for the detailed characterization of hCTR1. In summary, we have shown that hCTR1, the protein responsible for mediating copper uptake into human cells, has an extracellular amino terminus and a cytoplas-
mic carboxyl terminus (see Fig. 8). Mutation of the two putative N-linked glycosylation sites or the two native cysteine residues has no significant effect on copper uptake in Sf9 cells. Mutagenesis reveals Asn-15 as a glycosylation site for hCTR1. Additionally, our data suggest that the intracellular carboxyl terminus may be involved in forming stable multimers. This protein has few of the typical consensus metal-binding sequences associated with copper-dependent proteins, and the heterologous expression of this protein in Sf9 cells offers the opportunity to begin to address important questions that relate to the mechanism of copper entry into mammalian cells and the organization as well as structure-function relations in hCTR1.

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