O-Linked Glycosylation at Threonine 27 Protects the Copper Transporter hCTR1 from Proteolytic Cleavage in Mammalian Cells*

Received for publication, March 1, 2007, and in revised form, May 7, 2007 Published, JBC Papers in Press, May 24, 2007, DOI 10.1074/jbc.M701806200

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The major human copper uptake protein, hCTR1, has 190 amino acids and a predicted mass of 21 kDa. hCTR1 antibodies recognize multiple bands in SDS-PAGE centered at 35 kDa. Part of this increased mass is due to N-linked glycosylation at Asn-15. We show that in mammalian cells the N15Q mutant protein trafficked to the plasma membrane and mediated copper uptake at 75% of the rate of wild-type hCTR1. We demonstrate that the extracellular amino terminus of hCTR1 also contains O-linked polysaccharides. Glycosidase treatment that removed O-linked sugars reduced the apparent mass of hCTR1 or N15Q mutant protein by 1–2 kDa. Expression of amino-terminal truncations and alanine substitution mutants of hCTR1 in HEK293 and MDCK cells localized the site of O-linked glycosylation to Thr-27. Expression of alanine substitutions at Thr-27 resulted in proteolytic cleavage of hCTR1 on the carboxyl side of the T27A mutations. This cleavage produced a 17-kDa polypeptide missing approximately the first 30 amino acids of hCTR1. Expression of wild-type hCTR1 in mutant Chinese hamster ovary cells that were unable to initiate O-glycosylation also resulted in hCTR1 cleavage to produce the 17-kDa polypeptide. The 17-kDa hCTR1 polypeptide was located in the plasma membrane and mediated copper uptake at about 50% that of the rate of wild-type hCTR1. Thus, O-linked glycosylation at Thr-27 is necessary to prevent proteolytic cleavage that removes half of the extracellular amino terminus of hCTR1 and significantly impairs transport activity of the remaining polypeptide.

Copper is an essential trace element, acting as a catalytic cofactor for proteins involved in functions such as oxidative phosphorylation, detoxification of free radicals, iron uptake, neuropeptide synthesis, and connective tissue formation (1). Free Cu(1+), or Cu(2+) ions are not present in cells or in the serum in mammals, presumably because copper ions can participate in the formation of toxic reactive oxygen species (2, 3). The regulation of cellular copper levels involves uptake transporters, Cu-activated ATPases that mediate copper efflux, and several protein-specific chaperones that deliver copper to its intracellular target proteins (4–8).

CTR2 transport proteins constitute a major pathway of copper entry into eukaryotic cells. CTR homologs are found throughout eukaryotes, but they were first identified and studied in Saccharomyces cerevisiae (9). Yeast strains with deletions in the two high affinity copper uptake genes (ctr1 and ctr3) have a growth defect due to copper deficiency (9). Functional complementation of this growth defect was used to clone hCTR1, the human homolog of yeast CTR1 (10). hCTR1 is expressed in most if not all cell types (10). In mammals, CTR1 is an essential high affinity copper transporter, since mCTR1 homozygous knock-out animals die early in embryogenesis (11, 12).

CTR proteins contain three membrane-spanning segments, an extracellular amino terminus, a cytoplasmic loop between the first and second membrane spanning helices, and a cytoplasmic carboxyl-terminal tail (Fig. 1). A two-dimensional crystal structure of lipid-embedded hCTR1 was recently solved to 6 Å of resolution using electron crystallography (13), revealing a homotrimeric complex having a central pore. The copper transport activity of hCTR1 has been studied using 64Cu uptake in cultured cells. 64Cu uptake assays have been widely used to measure kinetic parameters of copper transport by hCTR1 and in structure-function studies of the protein (14, 15).

The extracellular amino terminus of hCTR1 may play a role in delivering copper ions from copper-binding proteins or copper complexes such as Cu(1+)-histidine to the transport pathway (7). The amino terminus has also been shown to self-associate, which may contribute to the stability of the trimeric complex (16). The amino termini of all CTR proteins contain conserved methionine- and histidine-rich motifs that might serve to focus the ions into the transport pathway (14). hCTR1 contains histidine- and methionine-rich motifs in the first 45 amino acids (Fig. 1A, H-I, H-2, and M-1, M-2, respectively). Mutagenesis studies showed that alanine substitutions and deletions affecting M-2 have the greatest effect on 64Cu uptake. Specifically, substitution of M43 and M45 (Fig. 1A, stars) dramatically reduced 64Cu uptake (15). Among hCTR1 mutants stably expressed in insect cells, amino-terminal truncations of the first 53 and 69 amino acids have substantially reduced 64Cu uptake, whereas a truncation of the first 34 amino acids had little effect (14).

It has repeatedly been observed that hCTR1 forms multimeric hCTR1 species, particularly dimeric forms, that are sta-

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2 The abbreviations used are: CTR1, copper transport protein; HEK293, human embryonic kidney 293; DMEM, Dulbecco’s modified Eagle’s Medium; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; tet, tetracycline; PNGase, glycosidase; MDCK, Madin-Darby canine kidney; IP, immunoprecipitation; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
Figure 1. hCTR1 protein. A, topology and domains of hCTR1. Shaded boxes in the extracellular amino-terminal domain represent histidine- and methionine-rich sequences common to CTR1 homologs. Starred methionines in M2 are essential for copper transport. The single site of N-linked polysaccharide addition is shown at N15 (N-Gly). B, detection of hCTR1 by Western analysis using antibodies (ab) against the cytoplasmic loop and (cytoplasmic) COOH-tail. Plasma membrane protein from Caco-2 cells (lanes 1 and 2, 30 μg), HEK293 cells (lane 3, 40 μg), and HEK293 cells overexpressing hCTR1 (lane 4, 10 μg) were detected with antibodies indicated below the panels. The 38-kDa band in lanes 2-4 detected by the anti-COOH tail antibodies is a cross-reacting protein unrelated to hCTR1. C, reciprocal immunoprecipitation using affinity purified anti-loop and anti-COOH tail antibodies. Endogenous hCTR1 was precipitated from solubilized Caco-2 membranes with anti-COOH tail antibodies and protein G-Sepharose (middle panel) or anti-cytoplasmic loop antibody coupled to beads (right panel). Light chain (L.C.) IgG from precipitating antibody is indicated.

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ble in denaturing polyacrylamide gels (15, 17, 18). The mobility of hCTR1 in gels is further complicated by the presence of glycosylation(s) in the amino terminus. The predicted molecular mass of hCTR1 is 21 kDa (10), but hCTR1 migrates with a mass of 33–35 kDa, often appearing as a closely spaced smear of bands (17, 19). Treatment of (mammalian) cells with tunicamycin or digestion with the N-linked polysaccharide-specific peptide glycosidase PNGase F reduces the apparent mass of hCTR1 to about 24–26 kDa (17, 20).

Two studies identified the site of N-linked glycosylation in hCTR1 at N15, confirming that the amino terminus is extracellular (17, 20). In human H441 cells, overexpressed N15D hCTR1 protein had a similar half-life and exhibited a similar immunofluorescence staining pattern in the plasma membrane as did overexpressed wild-type hCTR1 (16). When wild-type and N15Q hCTR1 proteins were overexpressed in insect cells, both proteins were abundant in plasma membrane fractions, and insect cells expressing N15Q had similar 64Cu uptake activity as cells expressing wild-type hCTR1 (14). Equivalent trafficking or 64Cu uptake experiments using N15Q hCTR1 expressed in mammalian cells have not been reported.

In the present work we have examined the consequences of removing N-linked polysaccharides from hCTR1 in mammalian cells and shown that removal of N15-linked glycosylation by mutation (N15Q) does not affect trafficking of the transporter to the cell surface. Copper transport activity of N15Q mutant protein was diminished by about 25% in comparison to the wild-type protein.

Previous work suggested that hCTR1 might also contain O-linked polysaccharides. In pulse-chase studies, endogenous hCTR1 in HeLa cells matured from a 28-kDa polypeptide to a 35-kDa form (17). In the presence of the N-glycosylation inhibitor tunicamycin, hCTR1 was synthesized as a 23-kDa precursor that chased into a species estimated as 30 kDa (17). Pulse-chase studies using N15D mutant hCTR1 also showed that a precursor 23-kDa protein chased into a 30-kDa species (16). The authors speculated that this 30-kDa species could be the result of O-linked glycosylation. Furthermore, endogenous hCTR1 protein from HEK293 cells treated with PNGase F and a mixture of glycosidases that remove O-linked sugars exhibited a greater increase in mobility than hCTR1 treated with PNGase F alone.3

In this report we establish that hCTR1 is O-glycosylated in mammalian cells, and we demonstrate that glycosylation takes place at Thr-27 in the extracellular amino terminus. Strikingly, we found that eliminating the O-linked glycosylation of hCTR1 results in essentially complete proteolytic cleavage of hCTR1 on the carboxyl side of Thr-27. The resulting 17-kDa polypeptide lacks approximately 30 amino acids from the amino terminus. The truncated hCTR1 protein is localized in the plasma membrane and has copper transport activity that is 50–60% that observed for wild-type hCTR1. It, thus, appears that O-linked glycosylation at Thr-27 of hCTR1 is necessary to protect the extracellular amino terminus from proteolytic removal from the transporter.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—HEK293 FLp-In™T-Rex™ cells and Madin-Darby canine kidney (MDCK) FLp-In™ T-Rex™ cells were cultured in Dulbecco’s minimal

3 J. Eisses and J. Kaplan, unpublished information.
**Glycosylation of hCTR1**

**TABLE 1**

| Oligonucleotide | Sequence, 5’–3’ |
|-----------------|----------------|
| Agel adaptor-sense | GATCCACACACCCGGTATGGATCATTCCCACCA |
| Agel adaptor-anti-sense | TATGGTGGAAAGTGGTGGCATGTCATGTTCG |
| FLAG-sense | GATCCACCGTGGCTTTCACCCACCCGACCC |
| FLAG-anti-sense | GAAGATGGTGGCTGCGTGGCTTCAGCATTTC |
| H22-sense | AATACGCCACGGTGGCTGGGTGATGGTGAG |
| A29-sense | AATACGCCACGGTGGCTGGGTGATGGTGAG |
| H22 and A29-anti-sense | GAAGATGGTGGCTGCGTGGCTTCAGCATTTC |
| ATS-sense | AAA-antisense |
| ATGAGGGATGTGAGGCTGCAGCGGCTGGGTGATGGTGAG |
| TAS-sense | AAA-sense |
| CATGGGAGTGTGAGGCTGAAGCGGCTGGGTGATGGTGAG |
| TTA-sense | AAA-antisense |
| CATGGGAGTGTGAGGCTGAAGCGGCTGGGTGATGGTGAG |
| TAA-sense | AAA-sense |
| CATGGGAGTGTGAGGCTGAAGCGGCTGGGTGATGGTGAG |
| TAS-antisense | AAS-antisense |
| CTACCATACCCACGCGCTGCAGCCTCACACTCCCATG |
| ATS-antisense | AATGAGGGATGTGAGGCTGCAGCGGCTGGGTGATGGTGAG |
| ATS-sense | AAS-sense |
| ACTGCAATCGATAAGGCCACGC |
| FLAG-antisense | ATAACCGGTCACCATCACCCAACCACTTCAGCC |
| GATCCACCGTGGCTTTCACCCACCCGACCC |

essential media (DMEM (Invitrogen catalog #11995), 25 mM Hepes buffer, and 10% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA). Caco-2 colorectal adenocarcinoma cells were cultured in DMEM/Hepes supplemented with 20% fetal calf serum. Chinese hamster ovary (CHO) cells, LLRDK (CHO) cells, and LLRD cell lines stably expressing hCTR1 were cultured in Ham’s F-12 media (Invitrogen catalog #11765) with 10% fetal calf serum and 100 μg/ml zeomycin (Invitrogen) or supplemented as described. All cells were grown at 37 °C in 5% CO₂. Cells were passaged every 3–7 days. HEK293 FLp-InTMT-RexTM cells and MDCK T-RexTM cells using the FLp-InTMT-RexTM core kit purchased from Biopeptide Co., LLC (San Diego, CA). Caco-2 colorectal adenocarcinoma cells were cultured in DMEM/Hepes supplemented with 20% fetal calf serum and 100 μg/ml tetracycline (Invitrogen) for 48 h before harvesting.

**hCTR1 Expression Constructs and Mutants**—The wild-type hCTR1 cDNA clone used here was obtained from Dr. J. Gitschier, UCSF GenBank™ accession number U83460. An Agel site was added 5’ of the initiating methionine codon by ligation of annealed oligos (Agel adaptor, Table 1) between the 5’ BamHI site and the Ndel site at nucleotide +16 in the hCTR1 coding sequence. A FLAG epitope tag was added to the amino terminus by ligation of annealed oligos between BamHI and Agel (NT-FLAG, Table 1) to produce the clone pEM79. This amino-terminal FLAG tagged hCTR1 cDNA was ligated into the FLp-In™ vector pcDNA5/FRT/TO© (Invitrogen) as a BamHI-Apal fragment to produce pEM83. The same BamHI-Apal fragment was ligated into pcDNA3.1/Zeo (+) (Invitrogen) to produce pEM89 for creation of stable hCTR1-expressing LLRD cell lines.

The NT-FLAG-tagged H22 and A29 truncation mutants were created from wild-type hCTR1 cDNA using PCR primer pairs listed in Table 1. hCTR1 genes lacking the first 21 (H22) or 28 (A29) amino acids were amplified with PCR, cut with Agel and Clal, and ligated into pEM83, replacing the full-length coding sequence with the truncated genes. Two truncation mutants (not FLAG-tagged) lacking the first 33 (MG34) or 52 (MN53) amino acids were amplified using oligos previously described (14) and ligated as AffII-EcoRI fragments into pcDNA5/FRT/TO©.

The NT-FLAG-tagged wild-type hCTR1 was transferred from pEM83 to the pBSIIKS vector (Stratagene, La Jolla, CA) as a BamHI-Apal fragment to produce pEM94. The N15Q mutation was introduced into pEM94 to produce pEM95 using the QuikChange® II site-directed mutagenesis kit (Stratagene) with the N15Q oligos in Table 1. pEM94 and pEM95 were used as templates to produce a set of alanine substitution mutants in amino acids Thr-26, Thr-27, and Ser-28 of hCTR1 (+809–817 in the hCTR1 cDNA). The wild-type TTS sequence was changed to ATS, TTA, TAA, and AAA in both pEM94 and pEM95 using the site-directed mutagenesis kit with mutagenic oligos listed in Table 1. The mutant hCTR1 genes were transferred as BamHI-Xhol fragments to pcDNA5/FRT/TO©. All clones were sequenced before use in experiments.

Restriction enzymes and T4 DNA ligase was purchased from New England Biolabs (Beverly, MA). PfuTurbo® polymerase for PCR was purchased from Stratagene. Oligos were purchased from Invitrogen, and DNA sequencing was performed by the Research Resources Center at the University of Illinois at Chicago.

**Antibodies and Affinity Purification**—The affinity-purified rabbit anti-hCTR1 antibody raised against the carboxyl-terminal peptide (SWKKKAVVVDITEHCH) was described previously (20) and used at 1/10,000 dilution. The anti-hCTR1 antibodies described in this work were raised against a 46-amino acid peptide corresponding to the cytoplasmic loop (KIARESVNLRTKSVSVSINMPVPGPNTGILMETHKTVGQQMLSFPH) purchased from Biopeptide Co., LLC (San Diego, CA).
This peptide was used to immunize rabbits (Cocalico Biologicals, Reamstown PA). Pre-immune and immune sera were tested for reactivity against hCTR1. The antibody was used at 1/1000 dilution.

hCTR1 cytoplasmic loop peptide was coupled to Actigel ALD resin matrix using the manufacturer’s instructions (Sterogene, Carlsbad CA). Post-coupled resin was packed into columns and used to purify anti-hCTR1 antibody from whole rabbit sera. Bound antibody was eluted with ActiSep elution medium (Sterogene), which was subsequently removed by desalting into PBS. The antibody was concentrated in spin concentrators (Vivasience AG, Hanover, Germany). Anti-hCTR1 loop IgG or normal rabbit IgG (Jackson ImmunoResearch Laboratories) was coupled to Actigel ALD resin for pull down reactions. IgG was purified from anti-hCTR1 loop whole serum using the Melon Gel™ system (Pierce) as suggested. Purified IgGs were coupled to Actigel as above.

Membrane Preparation— Cultured cells were washed twice in PBS and harvested from 10- or 15-cm plates by scraping. Cells were homogenized in the presence of a mixture of protease inhibitors (Roche Applied Science). Homogenization was done in a tight-fitting Dounce homogenizer followed by passing the lysate 3 times through a 27-gauge needle. Large debris was cleared by centrifugation at 1200 \(\times g\). The resulting microsomal supernatant was spun at \(\approx 90,000 \times g\) to pellet “total” membranes or layered in 5-step gradients to recover fractions enriched for plasma membranes, Golgi complex membranes, or membranes from the endoplasmic reticulum as described (21). Membrane protein concentration was determined using the method of Bradford (22).

Immunoprecipitation (IP)—IP and pulldown experiments in Fig. 1 were performed using membranes solubilized in \(n\)-dodecyl-\(\beta\)-D-maltoside (RPI Corp.). Membranes were resuspended in 0.1 M phosphate, pH 7.2, 150 mM NaCl, 5 mM dithiothreitol, and 1% \(n\)-dodecyl-\(\beta\)-D-maltoside for 45–60 min at room temperature. Samples were spun at \(\approx 90,000 \times g\) for 25 min at 4 °C, then the supernatant was removed. Supernatants were diluted into IP/pulldown buffer (50 mM phosphate, pH 7.2, 200 mM NaCl, 2.5 mM dithiothreitol, and 0.5% \(n\)-dodecyl-\(\beta\)-D-maltoside). IP reactions were precleared with protein G-agarose beads (Pierce). Precleared supernatant was collected by centrifugation at 1200 \(\times g\) for 5 min. For IPs, antibodies were added to precleared supernatant at 1/100 and rotated at 4 °C for 30–60 min, after which 10 \(\mu\)l of protein G-agarose beads were added, and the mixture was rotated overnight at 4 °C. Pulldown supernatants were precleared with normal rabbit IgG resin, and the supernatants were collected as for IPs. 50 \(\mu\)l of anti-hCTR1 loop IgG resin was then added to the supernatant, and the mixture was rotated overnight at 4 °C. IP beads or IgG resin was washed 5 times in IP/pulldown buffer, and bound proteins were eluted at 37 °C with 2× SDS electrophoresis sample buffer (125 mM Tris, pH 6.8, 2 mM EDTA, 6% SDS, 20% glycerol, 0.25% bromophenol blue, and 1% \(\beta\)-mercaptoethanol).

Cell Surface Biotinylation—HEK293 cells were surface-biotinylated with a reversible (thiol-cleavable) reagent (Sulfo-NHS-SS-Biotin, Pierce catalog #21331) as described (23). The cells were washed from 10-cm plates with DMEM media without fetal calf serum and spun 10 min at 800 \(\times g\) at 4 °C. Pellets were washed twice in PBS, and the cells were biotinylated while rotating for 25 min at 4 °C and quenched in buffers as described (23). Washed cells were then solubilized in 1% Triton-X-100 for 60 min, and insoluble material was removed by centrifugation at 12,000 \(\times g\). Supernatants were divided in equal portions, and half was incubated overnight at 4 °C with 100 \(\mu\)l of streptavidin beads (Pierce) that were equilibrated in solubilization buffer. The beads were collected and washed as described (23), after which the proteins bound to the beads were cleaved with 50 mM dithiothreitol in 2× SDS electrophoresis sample buffer at 30 °C before SDS-PAGE. The remaining half of the solubilized, biotinylated proteins were incubated with 200 \(\mu\)l anti-hCTR1 loop antibody-coupled beads in pulldown reactions as described above. The supernatants from streptavidin bead pulldown assays were subsequently incubated with the anti-hCTR1 beads in a second pulldown reaction. A control pulldown experiment using solubilized hCTR1 proteins from cells that were not biotinylated was performed using the same streptavidin and anti-hCTR1 beads. Preincubation with the streptavidin beads did not reduce the yield of unbiotinylated hCTR1 using anti-hCTR1 loop antibody beads (not shown).

PAGE and Western Blots—12 or 15% SDS-PAGE was performed using the method of Laemmli (24). Gels were transferred to Immobilon-P membranes (Millipore, Bedford MA) in 0.1 M CAPS buffer, pH 11, dried, and blocked with PBS containing 5% powdered milk and 0.1% Tween 20 (Fisher). Membranes were incubated with primary and secondary antibodies in the same solution and washed after incubations in PBS, 0.1% Tween. Western blot signals were obtained using SuperSignal®West reagents (Pierce) and collected by exposing to film or with a Chemi-Doc XRS system (Bio-Rad). Relative band intensity was determined using Quantity One® Software (Bio-Rad).

Glycosidase Treatment—Membranes were prepared for digestion with various glycosidases by partial denaturation with solutions provided by suppliers. Denaturation of hCTR1 samples was done for 10 min at 37 °C (higher temperatures aggregated the protein). Detergent and glycosidases were subsequently added to the reactions, and digestions were done at 37 °C for 2–3 days. Reactions were stopped with the addition of SDS sample buffer (above) before PAGE analysis. Asparagine-linked polysaccharides were removed from membrane samples with PNGase F (New England Biolabs). Threonine/serine-linked polysaccharides were removed with 1) a mixture of glycosidases included in the PRO-Link Extender™ kit (Prozyme, San Leandro, CA) or 2) using individual enzymes in the kit or purchased separately, including \(\alpha\)-2-fucosidase, \(\beta\)-N-acetylhexosaminidase, and Neuraminidase (New England Biolabs), and \(\beta\)-1–4-galactosidase (Prozyme).

\(^{64}\)Cu Uptake Assays—\(^{64}\)Cu uptake assays were similar to those described previously in insect cells (20), except that DMEM media containing 10% fetal bovine serum was used (DMEM 10%), which was previously shown to have a much lower level of hCTR1-independent copper uptake in mammalian cells compared with the buffer used in insect cells (18). LDL cells for \(^{64}\)Cu uptake were grown in Ham’s F-12 media containing 3% dialyzed calf serum supplemented with or without galactose and or N-acetyl-galactosamine (Sigma) 72 h...
before the assay. HEK293 cells expressing wild-type or mutant hCTR1 were induced with tetracycline in 10% DMEM 48 h before the assay.

One day before the assays 12-well tissue culture plates were seeded with 6–8 × 10⁵ cells/well in 10% DMEM. The following day the cells were washed once with 10% DMEM (without antibiotics), then incubated in 10% DMEM containing 2.5 μM CuCl₂ and trace amounts of ⁶⁴Cu (0.5–2.0 × 10⁶ cpm/well) for 5 min at room temperature or 45 min at 37 °C. Copper uptake was stopped by removing ⁶⁴Cu and adding ice-cold buffer (150 mM NaCl, 5 mM KCl, 2.5 mM MgCl₂, 10 mM EDTA, 25 mM Hepes, pH 7.4), after which the cells were washed twice with cold buffer, then dissolved in 1 ml 0.1 N NaOH. Lysed cells were collected, and half the volume was counted in a Beckman LS 6500 scintillation counter. A portion of the remaining lysed cells was used for protein determination. Each condition was done in triplicate wells and averaged. ⁶⁴Cu uptakes from 5-min incubations were used for protein determination. Each condition was collected, and half the volume was counted in a Beckman LS 6500 scintillation counter. A portion of the remaining lysed cells was used for protein determination. Each condition was done in triplicate wells and averaged. ⁶⁴Cu uptakes from 5-min incubations were subtracted from 45-min incubations to correct for nonspecific binding.

To normalize expression between tet-induced cell lines, 30 μg of total membrane protein or 10 μg of plasma membrane protein from each line was analyzed on Western blots using anti-hCTR1 and anti-Na⁺K-ATPase antibodies as described (20). For loading controls, signals from either the α-subunit of the Na⁺K-ATPase pump (for plasma membranes) or the 38-kDa cross-reacting protein described in the following paragraph (for total membranes) were quantitated. hCTR1 wild-type and mutant signals were also quantitated, and copper uptake rates were normalized for differences in expression of hCTR1. The ⁶⁴Cu uptake rate shown for the tet-induced cell-line expressing wild-type hCTR1 was consistently 6–8 pm/mg of protein/min, and other rates were normalized with respect to this. The expression of N15Q mutant protein averaged 59% of the wild-type level, so uptake values for N15Q in Fig. 2 were increased by a factor of 1.7. Expression of the AAA/N15Q mutant protein averaged 110% of the wild-type level; therefore, the average uptake value for the AAA/N15Q mutant (see Fig. 7) was reduced by 9%. ⁶⁴Cu-uptake experiments in LDLD cells were done in stably transformed cell lines and were, therefore, not adjusted for expression.

RESULTS

Identification of hCTR1 on Western Blots—Initial studies of hCTR1 proteins using Western blots showed that hCTR1 migrated with higher than expected mass in SDS-PAGE (15, 17, 18, 20). Although the predicted molecular mass of hCTR1 is 21 kDa, the size of the protein on Western blots using various anti-hCTR1 antibodies or epitope tags has been reported as 24, 28, and 35 kDa and multimeric forms of higher molecular mass (17, 18, 25, 26). The presence of higher mass bands that correspond to dimer, trimer, and higher mass species further complicates the identification of hCTR1 in gels. We previously raised a polyclonal antibody against the intracellular carboxy-terminal tail of hCTR1 that recognized membrane proteins of 34–35 and 38 kDa in mammalian cells (Ref. 20, Fig. 1B, lanes 2–4). For the present work, we raised polyclonal antibodies against the intracellular loop that recognized the 34–35-kDa bands (loop, Fig. 1B, lane 1). Reciprocal immunoprecipitation and detection with anti-loop antibodies and anti-carboxy-terminal antibodies showed that both antibodies recognize the same 34–35-kDa protein(s) (Fig. 1C). These results confirmed that the 38-kDa protein identified by the carboxy-terminal antibody was a cross-reacting protein unrelated to hCTR1 and showed that the 34–35-kDa protein bands corresponded to the native hCTR1 protein.

N-Linked Glycosylation of hCTR1—A substantial part of the extra mass of hCTR1 is accounted for by N-linked glycosylation at asparagine 15 (16, 17, 20). Treatment of endogenous hCTR1 in Caco-2 cells with PNGase F reduced the mass of the smear of bands to about 26 kDa (Fig. 2A, lane 5). Mutation of asparagine 15 to glutamine (N15Q) reduced the mass of hCTR1 to the same size as PNGase F treatment (Fig. 2A, lanes 2 and 3). Migration of the N15Q mutant protein was not altered by treatment with PNGase F (not shown), confirming previous conclusions that N15 is the only site of N-linked glycosylation in hCTR1 (16, 20).

N15Q mutant hCTR1 exhibited normal trafficking and somewhat reduced copper transport in cultured mammalian cells. Total membranes from tet-regulated HEK293 cell lines that overexpressed wild-type or N15Q mutant hCTR1 were separated on five-step sucrose gradients to isolate fractions
enriched for plasma membrane, Golgi, and endoplasmic reticulum (Fig. 2B, see Ref. 21). Both wild-type and N15Q mutant hCTR1 proteins were found predominantly in the plasma membrane fraction, as was the α-subunit of the Na,K-ATPase, a plasma membrane marker (Fig. 2B). The cross-contaminating 38-kDa protein described above was most abundant in the Golgi and ER-enriched fraction (Fig. 2B), with smaller amounts sometimes co-fractionating with the plasma membrane (e.g. Fig. 2A, compare lanes 3–5).

In addition to fractionating with surface membrane proteins, N15Q protein was efficiently labeled at the cell surface with an impermeant biotin reagent (Fig. 2C). Biotinylated cells were solubilized as described under “Experimental Procedures.” One-half of the solubilized proteins was incubated with streptavidin beads and the other half with anti-hCTR1-loop beads. Both beads pulled down wild-type or N15Q mutant proteins, although the streptavidin beads did so more efficiently (Fig. 2, lanes ab and str in each panel). The supernatants from the streptavidin pulldown reactions were then subjected to a second pulldown experiment using the anti-hCTR1 loop beads. As shown in Fig. 2C, lanes marked str-ab, only a small fraction of either solubilized hCTR1 protein remained after incubation with streptavidin beads. We conclude from these experiments that most of the solubilized wild-type and N15Q mutant hCTR1 proteins (>95%) were biotinylated and, thus, present in the cell surface membrane.

As seen in Fig. 2C, overexpressed FLAG-tagged N15Q protein migrated in SDS-PAGE as a pair of bands. The proteins were more distinctly separated in 15% polyacrylamide gels (Fig. 3). Biotinylated cells were solubilized as described under “Experimental Procedures.” One-half of the solubilized proteins was incubated with streptavidin beads and the other half with anti-hCTR1-loop beads. Both beads pulled down wild-type or N15Q mutant proteins, although the streptavidin beads did so more efficiently (Fig. 2, compare lanes ab and str in each panel). The supernatants from the streptavidin pulldown reactions were then subjected to a second pulldown experiment using the anti-hCTR1 loop beads. As shown in Fig. 2C, lanes marked str-ab, only a small fraction of either solubilized hCTR1 protein remained after incubation with streptavidin beads. We conclude from these experiments that most of the solubilized wild-type and N15Q mutant hCTR1 proteins (>95%) were biotinylated and, thus, present in the cell surface membrane.

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As seen in Fig. 2C, overexpressed FLAG-tagged N15Q protein migrated in SDS-PAGE as a pair of bands. The proteins were more distinctly separated in 15% polyacrylamide gels (Fig. 2C) than in 12% gels (Fig. 2, A and B). This pair of bands corresponded to epitope-tagged protein and protein that has lost the FLAG tag, presumably by proteolytic activity in vivo or during preparation (shown in Fig. 2).

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As seen in Fig. 2C, overexpressed FLAG-tagged N15Q protein migrated in SDS-PAGE as a pair of bands. The proteins were more distinctly separated in 15% polyacrylamide gels (Fig. 2C) than in 12% gels (Fig. 2, A and B). This pair of bands corresponded to epitope-tagged protein and protein that has lost the FLAG tag, presumably by proteolytic activity in vivo or during preparation (shown in Fig. 2).

As seen in Fig. 2C, overexpressed FLAG-tagged N15Q protein migrated in SDS-PAGE as a pair of bands. The proteins were more distinctly separated in 15% polyacrylamide gels (Fig. 2C) than in 12% gels (Fig. 2, A and B). This pair of bands corresponded to epitope-tagged protein and protein that has lost the FLAG tag, presumably by proteolytic activity in vivo or during preparation (shown in Fig. 2).

As seen in Fig. 2C, overexpressed FLAG-tagged N15Q protein migrated in SDS-PAGE as a pair of bands. The proteins were more distinctly separated in 15% polyacrylamide gels (Fig. 2C) than in 12% gels (Fig. 2, A and B). This pair of bands corresponded to epitope-tagged protein and protein that has lost the FLAG tag, presumably by proteolytic activity in vivo or during preparation (shown in Fig. 2).

As seen in Fig. 2C, overexpressed FLAG-tagged N15Q protein migrated in SDS-PAGE as a pair of bands. The proteins were more distinctly separated in 15% polyacrylamide gels (Fig. 2C) than in 12% gels (Fig. 2, A and B). This pair of bands corresponded to epitope-tagged protein and protein that has lost the FLAG tag, presumably by proteolytic activity in vivo or during preparation (shown in Fig. 2).

As seen in Fig. 2C, overexpressed FLAG-tagged N15Q protein migrated in SDS-PAGE as a pair of bands. The proteins were more distinctly separated in 15% polyacrylamide gels (Fig. 2C) than in 12% gels (Fig. 2, A and B). This pair of bands corresponded to epitope-tagged protein and protein that has lost the FLAG tag, presumably by proteolytic activity in vivo or during preparation (shown in Fig. 2).

As seen in Fig. 2C, overexpressed FLAG-tagged N15Q protein migrated in SDS-PAGE as a pair of bands. The proteins were more distinctly separated in 15% polyacrylamide gels (Fig. 2C) than in 12% gels (Fig. 2, A and B). This pair of bands corresponded to epitope-tagged protein and protein that has lost the FLAG tag, presumably by proteolytic activity in vivo or during preparation (shown in Fig. 2).

As seen in Fig. 2C, overexpressed FLAG-tagged N15Q protein migrated in SDS-PAGE as a pair of bands. The proteins were more distinctly separated in 15% polyacrylamide gels (Fig. 2C) than in 12% gels (Fig. 2, A and B). This pair of bands corresponded to epitope-tagged protein and protein that has lost the FLAG tag, presumably by proteolytic activity in vivo or during preparation (shown in Fig. 2).

As seen in Fig. 2C, overexpressed FLAG-tagged N15Q protein migrated in SDS-PAGE as a pair of bands. The proteins were more distinctly separated in 15% polyacrylamide gels (Fig. 2C) than in 12% gels (Fig. 2, A and B). This pair of bands corresponded to epitope-tagged protein and protein that has lost the FLAG tag, presumably by proteolytic activity in vivo or during preparation (shown in Fig. 2).

As seen in Fig. 2C, overexpressed FLAG-tagged N15Q protein migrated in SDS-PAGE as a pair of bands. The proteins were more distinctly separated in 15% polyacrylamide gels (Fig. 2C) than in 12% gels (Fig. 2, A and B). This pair of bands corresponded to epitope-tagged protein and protein that has lost the FLAG tag, presumably by proteolytic activity in vivo or during preparation (shown in Fig. 2).

As seen in Fig. 2C, overexpressed FLAG-tagged N15Q protein migrated in SDS-PAGE as a pair of bands. The proteins were more distinctly separated in 15% polyacrylamide gels (Fig. 2C) than in 12% gels (Fig. 2, A and B). This pair of bands corresponded to epitope-tagged protein and protein that has lost the FLAG tag, presumably by proteolytic activity in vivo or during preparation (shown in Fig. 2).

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Glycosylation of hCTR1

results showed that hCTR1 has O-linked polysaccharide(s) in addition to the glycosylation at N15.

To identify terminal residues of the O-linked polysaccharides on hCTR1, we treated plasma membranes from N15Q-expressing cells with individual exoglycosidases present in the mixture. In addition we also tested fucosidase alone and in the mixture (not shown). We found that sialidase was the only individual glycosidase that shifted the mobility of N15Q hCTR1 (Fig. 3C), indicating the presence of terminal sialic acid residues on a modest sized polysaccharide of between 1 and 2 kDa.

Localization of O-Linked Polysaccharides to Thr-26–Thr-27–Ser-28—We expected that the O-linked sugars would be located in the extracellular amino terminus of hCTR1, since the rest of the molecule consists of three transmembrane domains, a cytoplasmic loop, a short cytoplasmic carboxyl-terminal tail, and a short linker sequence between transmembrane domains two and three (Fig. 1). Because there are 16 serine and threonine residues distributed across the amino terminus of hCTR1 (Fig. 4A), we first tested four truncation mutants that lacked either the amino-terminal 22, 29, 34, or 53 amino acids to determine whether a shift in mobility occurred after treatment with the O-glycosidase mixture. We designed the H22 and A29 truncations to specifically test whether the Thr-26–Thr-27–Ser-28 sequence within this interval contained O-linked sugars. When the hCTR1 protein sequence was scanned using a predictive algorithm (29), Thr-26 and Thr-27 had the highest score for residues in hCTR1 likely to be sites of O-glycosylation (Fig. 4A, starred residues).

The various truncations together with wild-type and N15Q mutant hCTR1 were expressed in tet-regulated HEK293 and MDCK cell lines. The truncated hCTR1 proteins migrated in gels in descending order as more of the amino terminus was removed (Fig. 4B, HEK293 lines are shown). The wild-type, N15Q, H22, and A29 proteins included an amino-terminal FLAG tag, whereas the G34 and N53 proteins had only a methionine residue added to the amino terminus of the truncation. As seen above, wild-type and N15Q hCTR1 proteins were shifted to faster mobility after treatment with the O-glycosidase mixture. Of the four truncation mutants, only the H22 truncation was shifted by glycosidase treatment, showing that (at least part of) the O-linked glycosylation of hCTR1 occurred between H22 and A29 (Fig. 4B). The seven amino acids within this interval include Thr-26, Thr-27, and Ser-28. The size of the shift in mobility of the H22 mutant after glycosidase treatment appears comparable with that of wild-type or N15Q protein, but we could not definitively rule out the possibility that other residues on the amino-terminal side of H22 might also contain O-linked sugars.

Strikingly, the majority of the A29 truncation mutant protein underwent proteolytic cleavage on the carboxyl side of alanine 29. As shown in Fig. 4B the faster migrating protein in the A29 sample recognized by the carboxyl-terminal anti-hCTR1 antibody had an apparent mass of about 17 kDa. The 17-kDa A29 protein was recognized by loop and COOH-tail antibodies but was not recognized by anti-FLAG antibodies, showing that the FLAG-tagged amino terminus was lost on cleavage (Fig. 4C). The less abundant, slower migrating band was full-length, since it was recognized by both carboxyl-terminal and anti-FLAG antibodies (Fig. 4C).

The proteolytic cleavage near G34 was predominant only in the A29-truncated protein, although the H22 truncated protein contained variable amounts of the 17-kDa cleavage product (Fig. 4C, left and middle panels). A 17-kDa band of similar size was also seen at low levels in some hCTR1 preparations with
antibodies raised against the carboxyl terminus or intracellular loop of hCTR1. In any case, the A29 mutant protein was most susceptible to cleavage in multiple independent preparations from both HEK293 and MDCK cells. These preparations included other hCTR1 protein variants processed in parallel in the same buffers that were not cleaved.

**Substitutions of Thr-26, Thr-27, and Ser-28 Result in Proteolytic Cleavage**—We next identified the specific residue that was O-glycosylated by expressing mutants having single or multiple alanine replacements in the Thr-26–Thr-27–Ser-28 tripeptide sequence (TTS sequence). The mutations in TTS were made in both wild-type and N15Q mutant backgrounds. These mutants were in expressed in tet-regulated HEK293 and MDCK cells and were analyzed with Western blots to determine their state of glycosylation. We found that in both cell types, replacement of the TTS sequence with alanine residues (AAA) resulted in virtually complete proteolytic cleavage of hCTR1 protein very near the position cleaved in the A29 mutant (Fig. 5A). The AAS double substitution mutant protein was cleaved to nearly the same extent as the triple substitution AAA mutant, showing that one or both threonines are needed to protect against cleavage.

Further analysis showed that threonine 27 was the principle amino acid required within the TTS sequence to prevent cleavage. Three single substitution mutants (ATS, TAS, TTA) in the N15Q background were expressed in HEK293 and MDCK cells. Of these, the TAS mutant was most efficiently cleaved (Fig. 5B). We observed some cleavage of the ATS mutant protein, the extent of which was variable between preparations (Fig. 5B, lanes marked ATS). Multiple protein species observed between 24 and 34 kDa in Fig. 5B include 2 bands from uncleaved N15Q (seen in the N15Q TTS lane in Fig. 5A) and the dimeric form of the 17-kDa cleavage product, which migrated between the slower migrating uncleaved N15Q protein species and the wild-type hCTR1 smear at 34–35 kDa. This dimeric species was always observed to some extent in samples containing the 17-kDa protein but was never observed in samples that did not contain the 17-kDa species. M34 mutant protein was included in Fig. 5B to show the migration of the dimer from this protein, which is similar in size to the 17-kDa fragment.

It, therefore, appears that Thr-27 is the key site of O-linked glycosylation in the TTS sequence. Loss of O-glycosylation of hCTR1 caused by a lack of Thr-27 resulted in efficient proteolytic cleavage of hCTR1. This cleavage likely occurred on the carboxyl side of serine 28, since the FLAG-tagged A29 truncation mutant (Fig. 4) contained the site(s) of cleavage that resulted in the 17-kDa fragment. Because the MG34 truncation mutant migrated slightly faster than the 17-kDa cleavage product (Fig. 4C, ab panel, loop, *), the site of cleavage appears to occur between A29 and H33 in the sequence ASHSH.

**Loss of O-Linked Glycosylation in Wild-type hCTR1 Also Results in Proteolytic Cleavage**—We observed efficient proteolysis of hCTR1 when Thr-27 was substituted with alanine, and we inferred that the loss of O-linked glycosylation resulted in the cleavage of the hCTR1 amino terminus. It was also possible that alanine substitution mutations might affect the structure of hCTR1, rendering it susceptible to proteolysis due to misfolding (for example) and that the observed cleavage was not simply due to the loss of O-linked polysaccharides. We therefore expressed hCTR1 having wild-type sequence in CHO LDLD cells, which are conditional for O-glycosylation. LDLD cells are among a group of cell lines defective for the expression of low density lipoprotein receptors on their surface (30). LDLD cells lack epimerase activity needed for the synthesis of galactose and N-acetylgalactosamine. Under restrictive conditions in which N-acetylgalactosamine was not available, we found that cleavage of the N terminus of hCTR1 also occurred.

The expression level of CTR1 is very low in CHO cells and the LDL-CHO cell line.4 Therefore transfected LDL cells with amino-terminal FLAG-tagged wild-type hCTR1. Stable lines were selected in normal growth media and checked for hCTR1 expression with anti-FLAG and anti-hCTR1 antibodies (not shown). The level of hCTR1 overexpression was considerably less than hCTR1 expression in the tet-regulated HEK293 or MDCK cells, but hCTR1 was easily detected in purified plasma membrane fractions. To observe the effect of eliminating O-linked glycosylation on hCTR1, cells were grown with different sugars supplemented in a glycosylation-restrictive medium. The restrictive medium contained 3% dialyzed serum to eliminate contaminating galactose and N-acetylgalactosamine found in normal fetal calf serum. The four growth conditions tested had restrictive media supplemented with galactose and N-acetylgalactosamine, with galactose alone, with N-acetylgalactosamine alone, or with no added sugar. Plasma membrane fractions were prepared and examined on Western blots.

Western blot analysis revealed that hCTR1 was proteolytically cleaved when the LDLD cells were grown in the absence of N-acetylgalactosamine. As shown in Fig. 6, cells grown in the presence of galactose and N-acetylgalactosamine or N-acetylgalactosamine alone contained primarily full-length (34–35 kDa) hCTR1, whereas cells grown in galactose alone or no added sugar contained primarily the 17-kDa polypeptide. Some fully glycosylated 35-kDa protein synthesized before the 48-h

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4 E. B. Maryon and J. H. Kaplan, unpublished observation. 
treatment in restrictive media was probably still present, since the half-life of the 35-kDa hCTR1 protein was reported to be at least 2 days (16). In any case, hCTR1 is very efficiently cleaved in cells deprived of N-acetylgalactosamine, which are unable to initiate O-linked glycosylation (30). Perhaps surprisingly, the absence of galactose supplementation did not affect the migration of hCTR1 (Fig. 6, third lane), suggesting that galactose is not required for the maturation of either N- or O-linked polysaccharides on hCTR1. We conclude from these data and from the experiments above that in the absence of O-glycosylation at Thr-27, hCTR1 undergoes proteolytic cleavage to yield a 17-kDa polypeptide lacking ~30 amino acids from the amino terminus.

Proteolytically Cleaved hCTR1 Is Found in the Plasma Membrane—To determine the subcellular location of the 17-kDa hCTR1 fragment, we fractionated membranes from HEK293 and LDLD cells overexpressing wild-type or cleaved hCTR1. The HEK293 cell line used to examine the fractionation of the 17-kDa fragment contained the tet-inducible AAA/N15Q mutant hCTR1 (i.e. the triple substitution of alanine for Thr-26–Thr-27–Ser-28 that also included the N15Q mutation). As seen in Fig. 5A, most (greater than 95%) of the AAA/N15Q mutant protein is cleaved. Fractions enriched for plasma membrane, Golgi, and endoplasmic reticulum from HEK293 and LDLD CHO cells are shown in Fig. 7A. Both full-length and 17-kDa hCTR1 proteins were found greatly enriched in plasma membrane fractions. The α-subunit of the Na,K-ATPase, (a plasma membrane marker) co-fractionated with hCTR1 proteins (Fig. 7A).

To determine whether the 17-kDa hCTR1 fragment is in the plasma membrane and not simply co-fractionating with plasma membranes, we performed surface labeling in HEK293 cells expressing either wild-type or AAA/N15Q substitution mutant hCTR1 proteins. The cells were solubilized, divided in two equal portions, and incubated with either streptavidin-coated beads or beads linked to anti-hCTR1 loop antibodies. Proteins were eluted as described under “Experimental Procedures” and analyzed on Western blots.

As seen in Fig. 7B, both anti-hCTR1 (lanes ab) and streptavidin beads (lanes str) efficiently pulled down the wild-type and 17-kDa hCTR1 protein species. The location of the dimeric form of the 17-kDa polypeptides is noted (*). This species migrated slightly faster than did wild-type hCTR1 and was abundant in proteins solubilized with Triton-X-100. A small amount (less than 5%) of uncleaved protein (N15Q) is also visible in Fig. 7B, right panel (**). The supernatants from the streptavidin pulldown reactions were then subjected to a second pulldown experiment using the anti-hCTR1 loop beads. If wild-type or mutant proteins were not biotinylated (i.e. were not in the plasma membrane), they should be recovered by this second pulldown. Very little wild-type or 17-kDa protein was pulled down in this second reaction (Fig. 7B, lanes marked str-ab). Surface labeling of the AAS and TAS mutants gave similar results (not shown). We concluded from these experiments that the great majority (more than 95%) of wild-type and 17-kDa hCTR1 proteins were available for biotinylation and thus at the cell surface.

Proteolytically Cleaved hCTR1 Has Reduced Levels of Copper Transport—Having confirmed that the 17-kDa hCTR1 was located in the plasma membrane, we measured the capacity of this cleaved protein to transport copper. First, we measured the
level of copper transport in CHO cells and in LDLD-CHO cell lines stably expressing hCTR1. $^{64}$Cu uptake in the presence of 2.5 $\mu$M total copper was measured in cells grown in permissive or restrictive media for at least 72 h. Under these conditions the majority of hCTR1 in restrictive media underwent cleavage in LDLD cells (Fig. 7A). In LDLD cells overexpressing hCTR1 grown in the absence of galactose and $N$-acetylgalactosamine, $^{64}$Cu uptake was about 55% that of the level observed in cells grown with sugar supplementation (Fig. 7C). Sugar supplementation had no effect on $^{64}$Cu uptake in the parental CHO cell line, which has relatively low $^{64}$Cu uptake activity (Fig. 7C).

We then measured $^{64}$Cu uptake in HEK293 cells overexpressing wild-type or AAA/N15Q mutant hCTR1. The two cell lines were induced with tetracycline for 2 days and then assayed for $^{64}$Cu uptake in the presence of 2.5 $\mu$M copper. Uninduced control cells were also assayed under the same conditions. The results of this experiment are shown in Fig. 7D. Cells expressing the truncated 17-kDa hCTR1 protein (the AAA/N15Q mutant) mediated Cu transport at 50–60% that of the rate observed for full-length hCTR1. We also examined the trafficking and $^{64}$Cu transport capacity of hCTR1 that lacks $N$-linked glycosylation at Asn-15. N15Q mutant protein retained its native size, trafficked normally to the plasma membrane, and had about 75% copper transport activity of wild-type hCTR1.

**DISCUSSION**

We have identified a previously unknown modification of the amino terminus of the hCTR1 copper transporter, the addition of O-linked polysaccharides at Thr-27 (Fig. 8A). We show that in the absence of this glycosylation, the amino terminus undergoes a discrete proteolytic cleavage on the carboxyl side of Thr-27 to leave a truncated transporter that is located at the cell surface. The truncated hCTR1 protein mediated Cu transport at 50–60% that of the rate observed for full-length hCTR1. We also examined the trafficking and $^{64}$Cu transport capacity of hCTR1 that lacks $N$-linked glycosylation at Asn-15. N15Q mutant protein retained its native size, trafficked normally to the plasma membrane, and had about 75% copper transport activity of wild-type hCTR1.

**Anti-hCTR1 Antibodies**—The identification of hCTR1 on Western blots has been hampered by the tendency of the protein to migrate in SDS-PAGE as a multiplicity of bands of differing apparent molecular mass (17, 18, 25, 26). Several factors give rise to these numerous species, including variable post-translational modifications, multimeric and/or aggregated forms, proteolytic fragments, and irrelevant cross-reacting proteins. Using two independent antibodies raised against the carboxyl-terminal tail and the cytoplasmic loop (Fig. 1), we found that endogenous and overexpressed wild-type hCTR1 migrates in SDS-PAGE as closely spaced smear of bands at 33–35 kDa (Fig. 1). It is likely that the smear of full-length protein around 33–35 kDa is a consequence of $N$- and $O$-linked glycosylation. We used three lines of evidence to show that the 33–35-kDa species corresponds to monomeric hCTR1. First, two antibodies raised against different parts of hCTR1 recognize proteins of the same size in plasma membrane fractions from different cell types (Fig. 1B). Second, immunoprecipitations (or pulldown assays) using either of our two anti-hCTR1 antibodies precipitate 33–35-kDa proteins that are recognized by both antibodies. Third, both antibodies detect proteins of descending size in the expected order in plasma membranes from cells expressing a series of truncation mutants (Fig. 4).
**Glycosylation of hCTR1**

*N-Linked Glycosylation of hCTR1*—We examined the trafficking and $^{64}$Cu uptake activity of N15Q mutant hCTR1 in mammalian cells. In some cases, for example, the HERG voltage-gated potassium channel (31), N-linked glycosylation is required for trafficking to the surface membrane. We found that membranes containing overexpressed N15Q fractionated in an identical manner as wild-type hCTR1 (Fig. 2B) and that both N15Q and wild-type proteins were efficiently surface-labeled (Fig. 2C). However, the $^{64}$Cu uptake activity of N15Q was reduced by about 25% relative to wild type (Fig. 2D). Thus, although N-glycosylation of the transporter was not necessary for normal trafficking of hCTR1 to the surface, copper transport was somewhat reduced. N-Linked glycosylation may play a role in stabilizing or modifying the structure of hCTR1 in a way that facilitates copper transport. It is interesting to note that all mammalian CTR1 orthologs examined have a conserved NX(T/S) sequence in the amino terminus (Fig. 8B). 

*O-Glycosylation of hCTR1*—We showed that hCTR1 is O-glycosylated within the amino terminus, primarily if not exclusively at Thr-27. The loss of this glycosylation resulted in virtually complete cleavage of hCTR1 on the carboxyl side of Thr-27. The cleavage produced a 17-kDa polypeptide that was found in the plasma membrane and was functional as a copper transporter. This cleavage was observed in several different contexts in which O-linked glycosylation was absent (Figs. 4–6), and it occurred in human, canine, and Chinese hamster cell lines. We have not yet examined the glycosylation state of CTR1 proteins in non-human mammalian cells, but a comparison of the amino terminus from six sequenced orthologous CTR1 proteins shows that the TTS sequence and surrounding residues are largely conserved (Fig. 8B).

Based on glycosidase digests, the O-linked polysaccharide is 1–2 kDa, suggesting a single polysaccharide of 4–6 sugar residues that includes terminal sialic acid (Fig. 3C). Typical mucin-type polysaccharides share this general structure (27, 28). We infer that Thr-27 is the principle site of glycosylation based on the extent of cleavage of the Thr-27 alanine substitution mutant (TAS, Fig. 6). Because we also observe some cleavage in the Thr-26 mutant (ATS, Fig. 6), it is possible that Thr-26 is also glycosylated to some extent. However, it seems equally likely that the alanine substitution at Thr-26 may affect the efficiency of glycosylation of Thr-27 (32, 33). Further structural analysis of hCTR1 O-linked polysaccharides will be needed to confirm the identity and linkages of the monosaccharides as well as the specificity of glycosylation at Thr-27.

We could not determine from these experiments whether any of the 6 serine/threonine residues on the amino-terminal side of Thr-26 are also O-glycosylated because we have not yet isolated the amino-terminal fragment that results from cleavage. This fragment might be released into the medium or within the cell or be degraded, depending on the mechanism and location of cleavage. The shift (increase) in mobility of the H22 truncation mutant that contained the O-glycosylation at Thr-27 after glycosidase treatment appeared similar to that of the intact hCTR1 or N15Q protein (Fig. 4), suggesting that Thr-27 is the single site of O-linked polysaccharide addition in the transporter.

**Trafficking of hCTR1 to the Plasma Membrane**—In some instances O-linked glycosylation is necessary for efficient trafficking of membrane proteins to the cell surface (34, 35). We found that O-linked glycosylation of hCTR1 is apparently not required for trafficking of a functional transporter to the plasma membrane. First, expression of hCTR1 in LDL cells under conditions in which O-linked glycosylation does not occur did not prevent the trafficking of hCTR1 to the plasma membrane (Fig. 7, A and B). Second, in addition to various mutants lacking O-glycosylation, which were found in the plasma membrane as 17-kDa truncated proteins (Figs. 5 and 7), the G34 truncation mutant protein, which lacks both the Thr-27 and Asn-15, was competent for copper uptake and was found in plasma membrane fractions. However, we do not know if intact, unglycosylated hCTR1 is delivered to the plasma membrane and cleaved or if cleavage occurs before delivery.

It is conceivable that intact hCTR1 does not reach the surface unless it either becomes O-glycosylated or is cleaved, but the mechanism by which this would occur (as well as the compartment in which it would take place) is unclear. Furthermore, we have not observed intact un-O-glycosylated protein in Golgi- or ER-enriched membrane fractions (see Fig. 7A), so a parsimonious interpretation would be that O-linked glycosylation is not required for delivery of hCTR1 to the surface membrane. Thus, neither N- nor O-linked glycosylation appears to be required for normal trafficking of the transporter to the plasma membrane. It remains possible that hCTR1 glycosylation might affect trafficking of hCTR1 to apical or basolateral surfaces in polarized cells (36).

*Proteolytic Cleavage*—The cleavage of hCTR1 in the absence of O-glycosylation at Thr-27 produces a discreet 17-kDa polypeptide lacking the first 29–33 amino acids in hCTR1. We were able to map the cleavage to a site between A29 and G34 because the 17-kDa fragment had a slightly greater mass in SDS-PAGE than did the MG34 protein (Fig. 4C, *) and the A29 truncation mutant includes the cleavage site (Fig. 4B). Fig. 8A shows the deuced location of cleavage in hCTR1. This sequence, ASHSHG, is unusual because it does not contain conventional protease cleavage sites. This may indicate that the sequence is unusually exposed or vulnerable to general protease activity or that there is a protease associated with the hCTR1 transporter that specifically cleaves within this ASHSHG sequence.

We do not yet know the cellular compartment in which proteolytic cleavage of hCTR1 takes place. Because O-linked sugars are added in the Golgi stack at various points during maturation of proteins (27), it seems unlikely that cleavage would occur during transit through the Golgi compartment. The 17-kDa cleavage product was found in the plasma membrane fraction and was surface-labeled with biotin (Fig. 7). It seems most likely that cleavage occurs either late in the Golgi, during transit from the Golgi to the surface, within the plasma membrane, or in an endosome-like compartment that recycles to and from the surface. Numerous examples of membrane-associated or integral membrane proteases in the plasma membrane have been

5 S. Molloy and J. Kaplan, unpublished information.
reported (37–40) as well as examples of internalization and subsequent cleavage (41). We are currently in the process of investigating these issues.

The function of O-linked glycosylation of hCTR1 appears to be to protect against cleavage of a portion of the extracellular amino terminus. This role has been demonstrated for several other surface membrane proteins that contain O-linked polysaccharides, such as the low density lipoprotein receptor (30), the transferrin receptor (42), and the β-subunit of meprin (43). The need to prevent cleavage and subsequent loss of extra-cellular portions of these proteins may simply be to maintain the integrity of the protein structure, although in some cases (e.g. the transferrin receptor), cleavage after loss of O-linked glycosylation mimics a physiologic mechanism in which a soluble portion of the receptor is released by regulated cleavage (44).

In the case of hCTR1, the 17-kDa polypeptide is only partly impaired for copper transport in cell-based assays in vitro (Fig. 7), perhaps not surprising since the two methionines known to be essential for copper transport (15) are retained (Fig. 8A, starred residues). The portion of the amino terminus lost from the 17-kDa polypeptide is the most variable among CTR1 orthologs (Fig. 8B), but it includes the H-1 and H-2 histidine-rich and the M-1 methionine-rich regions (Fig. 1) that might have roles in vivo that are not evident in cell-based assays of function. The proteolysis of hCTR1 might represent an as yet uncharacterized aspect of the regulation of copper homeostasis in which the released glycopeptide binds to a target or that the remaining transporter has altered functions or both.

In summary, we have characterized the site of O-linked glycosylation of hCTR1, the major human copper uptake protein. The presence of this newly identified modification protects against a specific, efficient amino-terminal truncation by an as yet unknown protease. Whether or not there are hitherto unidentified regulatory or functional consequences of this proteolytic cleavage awaits further investigation.

Acknowledgments—We thank John Jellison for excellent technical assistance and Professor Anant Menon (Weill Cornell Medical College) and members of the Kaplan laboratory for helpful discussions.

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