Copper is an essential co-factor in many important physiological processes, but at elevated levels it is toxic to cells. Thus at both the organism and cellular level mechanisms have evolved to finely tune copper homeostasis. The protein responsible for copper entry from the circulation in most human cells is hCTR1, a small protein (190 amino acid residues) that functions as a trimer in the plasma membrane. In the present work we employ cell surface biotinylation and isotopic copper uptake studies of overexpressed hCTR1 in HEK293 cells to examine the acute (minutes) response of hCTR1 to changes in extracellular copper. We show that within 10 min of exposure to copper at 2.5 μM or higher, plasma membrane hCTR1 levels are reduced (by ~40%), with a concomitant reduction in copper uptake rates. We are unable to detect any degradation of internalized hCTR1 in the presence of cycloheximide after up to 2 h of exposure to 0–100 μM copper. Using a reversible biotinylation assay, we quantified internalized hCTR1, which increased upon the addition of copper and corresponded to the hCTR1 lost from the surface. In addition, when extracellular copper is then removed, internalized hCTR1 is promptly (within 30 min) recycled to the plasma membrane. We have shown that in the absence of added extracellular copper, there is a small but detectable amount of internalized hCTR1 that is increased in the presence of copper. Similar studies on endogenous hCTR1 show a cell-specific response to elevated extracellular copper. Copper-dependent internalization and recycling of hCTR1 provides an acute and reversible mechanism for the regulation of cellular copper entry.

Copper-dependent Recycling of hCTR1, the Human High Affinity Copper Transporter*

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Copper is an essential micronutrient and plays an important function as a co-factor for a number of cellular processes including oxidative phosphorylation, free radical detoxification, neurotransmitter synthesis, iron metabolism, and maturation of connective tissue (1). Copper in excess of cellular requirements is toxic; therefore cells have developed sophisticated mechanisms for regulating copper acquisition and secretion, thus maintaining a critical copper homeostasis (2, 3). In eukaryotes a family of transporters known as the copper transporter (Ctr) proteins mediate cellular copper uptake (4). Ctr proteins are integral membrane proteins that are structurally conserved with three membrane-spanning domains and a number of methionine rich motifs in the N terminus (5). They contain a sequence of conserved cysteine and histidine residues at or close to the C terminus and are predominantly located at the plasma membrane (6). In the yeast, Saccharomyces cerevisiae, the first high affinity copper transporters, yCtr1 and yCtr3, were identified (7, 8), and this facilitated the identification of the human copper transporter gene, hCTR1, by functional complementation of yeast high affinity copper uptake mutant, ctr1 (9). The mouse CTR1 is 92% identical to hCTR1 (10), and the deletion of mCTR1 results in early embryonic lethality, suggesting an essential role for the high affinity copper transporter in mammalian growth and development (11).

hCTR1 has 190 amino acid residues, three membrane-spanning domains, an extracellular N terminus (of 66 amino acids), a large cytoplasmic loop (of 46 amino acid residues), and a short C-terminal tail (of 15 amino acids) and has been shown to form stable dimers and trimers (12–14). The hCTR1 protein has been shown in 64Cu uptake experiments to mediate copper transport with a K_m of 1–5 μM and is thought to transport the reduced form, Cu(I) (12, 13, 15). The extracellular N terminus has both N- and O-linked glycosylation at residues Asn^{15} and Thr^{27}, respectively (12, 16, 17), and contains two histidine-rich regions and two methionine motifs that are thought to function in copper binding/sensing. Recent studies showed that mutation or deletion of the methionine residues closest to the first transmembrane domain (Met^{43} and Met^{45}) and the conserved methionine residues in the second transmembrane domain (Met^{150} and Met^{154}) had a large inhibitory effect on 64Cu uptake (18, 19). Mutational analysis provided no evidence for the tight binding of copper at any specific residues, and it was proposed that hCTR1 provided a pore for the permeation of copper across the membrane (18). Structural confirmation of such a mechanism was provided in the low resolution structure obtained by cryo-electron microscopy studies on recombinant protein (20, 21).

Considerable progress has been made in understanding the biochemical, structure-functional, and molecular aspects of hCTR1-mediated copper transport, although many questions remain unanswered (22). It is also important to determine whether or not hCTR1 has a regulatory role preventing the accumulation of toxic levels of copper and maintaining cellular copper homeostasis. Previous reports on whether or not hCTR1 is involved in an acute response to elevated copper have
been somewhat controversial. It has been reported that elevated extracellular copper (1–100 μM) stimulates rapid endocytosis and degradation of hCTR1-Myc-tagged protein in HEK293 cells (23), but also high copper levels had no effect on endogenous hCTR1 localization in both HeLa and Caco-2 cells (14). In a study of overexpressed hCTR1 in insect cells, no evidence was seen of internalization in response to elevated copper (24). Imaging studies have shown that the cellular location of hCTR1 varies among cell lines, CTR1 in MDCK and HEK293 cells resides mainly at the plasma membrane (13, 15, 23, 24). Endogenous hCTR1 is located in cytoplasmic vesicular compartments in HeLa, Caco-2, and HepG2 cell lines with some plasma membrane staining in Caco-2 (14). In intestinal sections, basolateral and subapical staining is seen (15).

Previous studies (see above) have utilized internalization of prebound antibody (23) or imaging methods (14) to characterize the response of hCTR1 to elevated copper. In the present work we employed HEK cells overexpressing hCTR1 and used cell surface biotinylation, a sensitive and quantitative measure of CTR1 at the cell surface (15, 17). We have combined this with measurements of hCTR1-mediated 64Cu uptake as a functional measure of plasma membrane hCTR1 levels. We find that a fraction (~40%) of hCTR1 is rapidly internalized in the presence of elevated copper and that there is a concomitant reduction in the hCTR1-mediated copper uptake rate. The internalized transporter is not degraded and can be detected in the cytosol. On removal of extracellular copper, the transporter is recycled promptly to the plasma membrane. Internalization of endogenous CTR1 is also observed in MDCK and HepG2 cells, and no reduction is seen in T47D cells. This is, to our knowledge, the first such report of copper-dependent recycling of hCTR1 in response to copper and represents an acute regulatory mechanism that reversibly modulates cellular copper entry.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—All of the cells were maintained in an incubator at 37 °C and 5% CO2. MDCK, HeLa, HepG2, and HEK293 Flp-InTM T-RExTM (Invitrogen) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 25 mM Hepes, and 1% PSF. The cells were passaged every 3–5 days.

HEK293 cells containing tetracycline-regulated N-terminal FLAG-tagged hCTR1 were created as previously described (17). Briefly the N-terminal FLAG-tagged hCTR1 was ligated into pcDNA5/FRT/TO vector (Invitrogen) and co-transfected with pOG44 plasmid (Invitrogen) using Lipofectamine 2000 (Invitrogen) into HEK293 Flp-InTM T-RExTM cells. Transfected cells were selected in medium containing 10 μg/ml blasticidin and 400 μg/ml hygromycin B, and hCTR1 protein expression was induced by the addition of 1 μg/ml of tetracycline to the medium for 48 h.

**Copper Treatment**—MDCK cells were grown on 24-mm polystyrene membrane transwells (Corning) with 0.4-μm pore size until confluent (15, 25); all other cells were grown in 10-cm Petriplates. The cells were pretreated with 100 μg/ml of cycloheximide for 30 min to inhibit new protein synthesis, after which CuCl2 was added at the required concentration and time to the growth medium; for MDCK cells copper was added to the medium in the basolateral compartment of the transwell.

**Biotinylation of Surface hCTR1**—Biotinylation of cells was carried out using the cell-impermeable, thiol- cleavable Sulfo-NHS-SS-biotin (Pierce) reagent to label cell surface proteins. All of the biotinylation procedures were carried out at 4 °C. Biotinylation of cells grown on monolayers was carried out essentially as described previously (17, 25). For MDCK cells grown in transwells either the apical (A) or basolateral (B) membrane was biotinylated (15, 25). Before biotinylation the cells were placed on ice, the growth medium was removed, and the cells were washed once with DMEM supplemented with 25 mM Hepes buffer and twice with PBS supplemented with 0.1 mM CaCl2 and 1.0 mM MgCl2. The cells were then incubated with 0.77 mg/ml of Sulfo-NHS-SS-biotin diluted in biotinylation buffer (10 mM triethanolamine, pH 7.5, 2 mM CaCl2, and 150 mM NaCl) for 25 min. The cells were then rinsed with quench buffer (PBS with 0.1 mM CaCl2, 1.0 mM MgCl2, and 100 mM glycine) and incubated with quench buffer for 20 min, and this step was then repeated. The cells were lysed in lysis buffer (1% Triton-X-100, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.5) and incubated rotating end-over-end for 1 h. The lysed cells were centrifuged at 10,000 × g for 10 min, and 5% of the supernatant was removed to determine protein concentration using the Bradford method (26). The remaining supernatant was placed in a spin column, to which 100 μl of streptavidin-agarose (Pierce) was added per 900 μl of supernatant and incubated overnight at 4 °C with end-over-end rotation. The samples were then centrifuged at 500 × g for 5 min and washed three times with lysis buffer, twice with salt wash buffer (0.1% Triton-X-100, 500 mM NaCl, 5 mM EDTA, and 50 mM Tris, pH 7.5), and once with no salt wash buffer (10 mM Tris, pH 7.5). The lysed cells were centrifuged at 10,000 × g for 5 min. The protein was eluted in 50–100 μl of 2× SDS-PAGE sample buffer with 150 mM dithiothreitol and separated by SDS-PAGE, and hCTR1 was detected using either anti-hCTR1 or anti-FLAG antibody by Western blot analysis as described below.

To evaluate the efficiency of precipitation of biotinylated protein using streptavidin-agarose beads, the supernatants from the initial precipitation were incubated with fresh streptavidin-agarose beads. The second treatment with beads showed no detectable biotinylated hCTR1, confirming the high efficiency of the initial treatment (result not shown).

hCTR1 has been shown to form SDS-stable multimers in the form of dimers and trimers (12–14) in gels. We also detected these higher order multimers in our experiments, and their levels decreased with increasing copper concentration (result not shown) just like the monomeric form of hCTR1 (see Fig. 1A). The dimeric and trimeric forms of hCTR1 together represented less than 10% of the total hCTR1 protein. We have routinely used the monomeric form of hCTR1 only in our quanti-
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tation of changes in hCTR1; inclusion of the multimers in the calculation had no significant effect on our observations.

Immunoprecipitation—Immunoprecipitation was performed essentially as described previously (17) except the cells were first biotinylated as described above using the noncleavable cell-impermeable Sulfo-NHS-LC-LC-biotin (Pierce). The solubilized biotinylated protein was added to anti-hCTR1 C-terminal antibody coupled to protein A beads and incubated overnight at 4 °C with end-over-end rotation. The bound proteins were eluted in 2× SDS-PAGE sample buffer with 1% β-mercaptoethanol and analyzed by SDS-PAGE, and biotinylated hCTR1 was detected using streptavidin-HRP (Pierce) and anti-hCTR1 loop antibody (17).

Copper-stimulated hCTR1 Internalization Quantitated by Reversible Biotinylation—hCTR1 internalization was measured by its protection from reductive cleavage by a membrane-impermeable reducing reagent, glutathione, which releases the bound biotin reagent from surface-accessible proteins (27). Initially the cells were biotinylated (as detailed above) at 4 °C. After washing twice with quench buffer, the cells were raised to 37 °C by the addition of growth medium, to which 20 μM CuCl₂ was added for incubation periods of 0–120 min to allow for any copper mediated hCTR1 endocytosis. Any endocytosis was stopped by placing the cells on ice, the growth medium was removed, and all subsequent steps were performed at 4 °C. The cells were washed twice with cold PBS, and cell surface biotin was cleaved by incubating the cells for 15-min intervals in cleavage buffer (50 mM glutathione, 90 mM NaCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 60 mM NaOH, and 0.2% bovine serum albumin) for a total of 45 min. After cleavage, the free SH groups on the cell surface were quenched by the addition of 5 mg/ml of iodoacetamide in PBS for 15 min. The cells were then solubilized in lysis buffer containing 1 mg/ml of iodoacetamide for 1 h and processed as described for surface biotinylation. The eluted protein was subject to SDS-PAGE, and hCTR1 was detected by Western blot analysis using either anti-hCTR1 or anti-FLAG antibodies as described below.

SDS-PAGE and Western Blot Analysis—Protein samples were separated using 15% SDS-PAGE and transferred to Immobilon-P membranes (Millipore) in 0.01 M CAPS buffer, pH 11, and blocked in PBS containing 5% milk. The membranes were either probed with rabbit anti-hCTR1 C-terminal antibody (12) or mouse anti-FLAG antibody (GenScript) followed by either donkey anti-rabbit HRP (GE Healthcare) or goat anti-mouse HRP (Thermo Scientific) secondary antibodies. Western blot signals were obtained using SuperSignal West reagents (Pierce) and developed using the Chemi-Doc XRS (Bio-Rad) detecting chemiluminescence. The signal intensity was quantified using Quantity One® software (Bio-Rad) and was shown to be within the linear range of detection. As a protein loading control the integral membrane protein Na,K-ATPase was detected and quantitated simultaneously on the immunoblot using the mouse anti-β1 antibody (28).

64Cu Uptake—Copper uptake assays were performed similar to those previously described (13, 17). One day prior to the assay, the cells were seeded onto 12-well tissue culture plates at a density of 0.7 × 10⁶ cells/ml/well in DMEM containing 10% fetal bovine serum and incubated overnight at 37 °C in a 5% CO₂ incubator. For the cells that were preincubated with copper, a range of copper concentrations was added to the growth medium, and the cells were incubated for the required time. After the preincubation the cells were washed twice with DMEM containing 10% fetal bovine serum and incubated with either 2.5 or 10 μM CuCl₂ containing 64Cu (Isotrace Technologies Inc.) for either 5 or 45 min at 37 °C. Copper uptake was halted by adding ice-cold stop buffer (150 mM NaCl, 5 mM KCl, 25 mM Hepes, pH 7.4, 2.5 mM MgCl₂, and 10 mM EDTA) to the cells, which were subsequently washed twice with stop buffer. The cells were lysed in 0.1 M NaOH, and an aliquot of the cell lysate was added to EcoLume Scintillation fluid and counted in a Beckman LS6500 scintillation counter. The protein concentration of the cell lysate was determined after radioisotope decay, and the copper uptake/well was calculated as pmol of Cu/mg of protein, and the average of triplicate wells was determined for each treatment. The 5-min 64Cu uptake was subtracted from the 45-min uptake value as a correction for binding, and the final 64Cu uptake result was calculated as pmol of Cu/mg of protein/min.

Total Membranes—The cells were washed twice with PBS and harvested from 15-cm Petriplates in PBS using a cell scraper. The cells were pelleted by centrifugation at 1,000 × g for 10 min at 4 °C, and the pellet was resuspended in homogenization buffer (0.25 M sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4) containing protease inhibitor mixture (Roche Applied Science). The cells were lysed on ice using a tight fitting Dounce homogenizer (20 strokes), and the lysate was then passed through a 27½-gauge needle. The lysed cells were centrifuged at 1,000 × g for 10 min at 4 °C to pellet large debris, and the resulting supernatant was centrifuged at 90,000 × g for 30 min at 4 °C. The membrane protein concentration was determined using the Bradford method (26).

Statistical Analysis—The results are presented as the means ± S.D. of three separate experiments. Statistical analysis was performed using PASW v17 (SPSS Inc.). A one-sample Kolmogorov–Smirnov test was used to determine whether the data were normally distributed. The statistical analyses are described in the figure legends, where p < 0.05 was determined as statistically significant.

RESULTS

Copper-stimulated Reduction of Plasma Membrane hCTR1 Levels—To detect and quantify changes in plasma membrane hCTR1 in response to copper, we employed cell surface biotinylation using an impermanent reagent adapted from previously described protocol (25) that has been shown to effectively label cell surface hCTR1 overexpressed in HEK293 cells (17).

We initially investigated the effect of increasing copper concentration on hCTR1 using HEK293 cells expressing N-terminal FLAG-tagged hCTR1. The cells were incubated for 30 min in medium supplemented with 100 μg/ml cycloheximide, after which copper ranging from 0 to 100 μM was added to the medium for 2 h. The cell surface proteins were then biotinylated (see “Experimental Procedures”) and harvested using avidin-conjugated beads. The surface level of hCTR1 was detected using either anti-FLAG or anti-hCTR1 C-terminal antibody. Both antibodies showed similar results (Fig. 1A). At the same
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For 2 h, Copper uptake assays were performed at 10 μM CuCl₂ and calculated as pmol copper/mg protein/min. The results shown here are the means ± S.D. for a single experiment and are representative of those obtained from three independent experiments. A one-way ANOVA with a Games-Howell post-hoc test was used to determine statistical significant differences. The asterisk denotes statistical significance (p < 0.05) compared with the control (no copper).
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As shown in Fig. 2, hCTR1 itself is clearly biotinylated.

**Time Course of hCTR1 Internalization**—It was of interest to determine how rapidly hCTR1 responded to changes in extracellular copper. The cells were treated with 2.5 or 20 μM copper for 0–120 min and then biotinylated. At both copper concentrations we detected a similar rate of reduction in plasma membrane hCTR1 and show the result at 20 μM copper (Fig. 3A). Quantitation of these blots is shown in Fig. 3B where surface hCTR1 levels fall rapidly (within 10 min) and are subsequently unaltered. These observations suggest that the copper-stimulated internalization of hCTR1 is a rapid phenomenon that involves a fraction (~40%) of the total surface hCTR1 molecules.

**Fate of Internalized hCTR1**—We have shown that hCTR1 plasma membrane levels decreased rapidly after exposure to copper. If hCTR1 is internalized in response to copper and degraded (23), there should be a reduction in the total cellular content of hCTR1. To examine this possibility, HEK293 cells expressing N-terminal FLAG-tagged hCTR1 were incubated for 30 min with 100 μg/ml cycloheximide, after which a range of copper concentrations were added to the cycloheximide-containing medium for 2 h. To detect any possible hCTR1 degradation at the N or C terminus, we analyzed Western blots of total cell membranes from copper-treated cells with the anti-hCTR1 C-terminal and anti-FLAG antibody, respectively, and the same blots were probed with β-subunit of the Na,K-ATPase as a loading control. We could detect no degradation products of lower mass derived from hCTR1 with either antibody and have shown the result with the FLAG antibody (Fig. 4A). The hCTR1 signal intensity was quantified, and the data were analyzed using one-way ANOVA, and there was no significant differences in the total membrane hCTR1 mean values in response to extracellular copper (Fig. 4B). This result shows that there is neither degradation of hCTR1 at the N or C terminus nor any change in total hCTR1 levels in response to extracellular copper.

**Detection of Internalized hCTR1**—We employed a reversible biotinylation method based on that used to detect internalized insulin-responsive aminopeptidase protein (27) to quantitate hCTR1 that had been internalized in response to extracellular copper. HEK293 cells overexpressing hCTR1 were biotinylated at 4 °C (see “Experimental Procedures”) and then incubated either with streptavidin-agarose to detect biotinylated cell surface hCTR1 or in basal medium at 37 °C in the presence of 20 μM copper. HEK/hCTR1-FLAG cells were incubated for 30 min with 100 μg/ml cycloheximide, after which 20 μM copper (Cu) was added to the cycloheximide-containing medium for 0–120 min, and the cells were then biotinylated. A, the biotinylated proteins were analyzed by Western blots, and hCTR1 protein was detected using anti-FLAG and anti-hCTR1 C-terminal antibody. Both FLAG and hCTR1 C-terminal antibodies showed similar detection results; only the Western blot probed with FLAG antibody is shown. B, the hCTR1 protein detected was quantified as described under “Experimental Procedures” and normalized to the Na,K-ATPase β1 subunit, and the values were expressed as a percentage of total cell surface hCTR1 at time 0 (no extracellular copper added). The graph represents the means ± S.D. of three separate experiments. The data were normally distributed, and a one-way ANOVA followed by a Dunnett post hoc test was used to determine significant differences (*, p < 0.05) between control (no copper) and copper-treated cells.

**FIGURE 2. Direct biotinylation of hCTR1.** HEK/hCTR1-FLAG and non-FLAG-tagged cells were labeled with cell impermeant noncleavable Sulfo-NHS-LC-LC-biotin. The solubilized proteins from labeled cells were incubated with anti-hCTR1 C-terminal antibody-coupled beads, immunoprecipitates (IP) were analyzed on Western blots and probed with either streptavidin-HRP or anti-hCTR1 loop antibody (Ab).

**FIGURE 3. Time-dependent decrease in cell surface hCTR1 protein in the presence of extracellular copper.** HEK/hCTR1-FLAG cells were incubated for 30 min with 100 μg/ml cycloheximide, after which 20 μM copper (Cu) was added to the cycloheximide-containing medium for 0–120 min, and the cells were then biotinylated. A, the biotinylated proteins were analyzed by Western blots, and hCTR1 protein was detected using anti-FLAG and anti-hCTR1 C-terminal antibody. Both FLAG and hCTR1 C-terminal antibodies showed similar detection results; only the Western blot probed with FLAG antibody is shown. B, the hCTR1 protein detected was quantified as described under “Experimental Procedures” and normalized to the Na,K-ATPase β1 subunit, and the values were expressed as a percentage of total cell surface hCTR1 at time 0 (no extracellular copper added). The graph represents the means ± S.D. of three separate experiments. The data were normally distributed, and a one-way ANOVA followed by a Dunnett post hoc test was used to determine significant differences (*, p < 0.05) between control (no copper) and copper-treated cells.
treatment none of the previously surface-labeled protein (lane 1) could be detected (lane 2), indicating that glutathione was effective in quantitatively removing biotin from surface-labeled proteins. After incubating the cells for 10 min in the presence of 20 μM copper, hCTR1 that has been internalized is readily detected (Fig. 5, lane 4). The internalized biotin-labeled protein was quantified and expressed as a percentage of the total surface hCTR1 in lane 1 (Fig. 5B). The amount of internalized hCTR1, ∼30%, agrees well with the loss of surface hCTR1 determined previously to be ∼38% (Fig. 3B) under similar conditions. This confirms the lack of significant degradation reported above, because the amount of hCTR1 lost from the surface is detected within the cell. As can be seen in Fig. 5B, a small amount, ∼14% of the total surface-labeled hCTR1 was intracellular in basal medium without added copper (Lane 3). The result in Fig. 5 indicates that the internalization of hCTR1 is copper-induced in keeping with our previous observations (see above). It is important to note that even after prolonged exposure (2 h) to extracellular copper, intact internalized hCTR1 is still detected, indicating that copper stimulates the internalization of hCTR1 but not to any significant extent its degradation.

Copper-induced Endocytosis and Return of hCTR1 to the Plasma Membrane—Menkes and Wilson disease proteins, which reside in the trans-Golgi network, undergo copper-dependent trafficking between the trans-Golgi network and post-trans-Golgi network compartments (30, 31). Because we had observed copper-dependent internalization of hCTR1 (without degradation), we wondered whether hCTR1 might also cycle between the plasma membrane and an intracellular compartment. We investigated the copper-induced endocytosis and potential recycling of hCTR1 back to the plasma membrane by
incubating HEK/hCTR1-FLAG cells in 20 μM copper for 10 min (which we previously showed induces hCTR1 internalization; Figs. 3 and 5) and then resuspended the cells in basal medium. This cycle was then repeated, and biotinylation was carried out after each solution change. The possible involvement of newly synthesized hCTR1 was avoided by the inclusion of cycloheximide (see “Experimental Procedures”). As shown in Fig. 6A (lane 2), the addition of 20 μM copper to the cells for 10 min reduced plasma membrane hCTR1 levels, and quantitation of the signal (Fig. 6B) showed a reduction of ~35% (similar to that shown previously in Fig. 3). After the removal of extracellular copper, surface hCTR1 returned to the level before copper addition (Fig. 6A, lane 3). A subsequent incubation in copper-containing medium again reduced the hCTR1 levels at the plasma membrane, indicating that copper stimulates the endocytosis of hCTR1 but that in the absence of copper, hCTR1 recycles back to the plasma membrane.

Kinetics of Recycling—How fast does the previously internalized hCTR1 return to the plasma membrane? To assess this, the cells were first subjected to copper-induced endocytosis by the addition of 20 μM copper for 10 min. The cells were incubated in basal medium for 0–30 min, biotinylated during this recovery phase, and examined by Western blot analysis. hCTR1 was detected using anti-FLAG and anti-hCTR1 C-terminal antibodies, and both showed similar results, C-terminal antibody data are shown (Fig. 7A). Following the 10 min of exposure to copper, plasma membrane hCTR1 returned to steady state levels within 30 min.

FIGURE 6. Copper-dependent internalization and recycling of hCTR1. HEK/hCTR1-FLAG cells were incubated: without copper (Cu, lane 1), with 20 μM copper for 10 min (lane 2), with 20 μM copper for 10 min followed by incubation in basal medium for 30 min (lane 3), or with 20 μM copper for 10 min in basal medium for 30 min followed by the addition of 20 μM copper for an additional 10 min (lane 4). The cells were biotinylated after each treatment as described under “Experimental Procedures.” A, the biotinylated protein was analyzed on Western blot, and hCTR1 was detected using anti-FLAG antibody. B, the hCTR1 protein detected in Western blots was quantitated using Quantity One (Bio-Rad), normalized to the β1 subunit, and expressed as a percentage of total cell surface hCTR1 at time 0, which was set to 100%. The down arrows indicate the addition of 20 μM copper to the cells for 10 min, and the up arrow indicates the removal of copper from the cells. The graph represents the means ± S.D. of three separate experiments, and the asterisk denotes statistically significant ($p < 0.05$) difference compared with the control (no copper pretreatment), using unpaired $t$ test.

FIGURE 7. Return of hCTR1 to the plasma membrane. HEK/hCTR1-FLAG cells were grown in basal medium, pretreated for 30 min with cycloheximide, and then incubated with 20 μM copper (Cu) for 10 min followed by incubation in basal medium for 0–30 min. The cells were biotinylated as described under “Experimental Procedures” at the indicated times. A, the biotinylated protein was analyzed by Western blot and detected using anti-hCTR1 C-terminal. B, the biotinylated hCTR1 protein detected in Western blots was quantitated using Quantity One (Bio-Rad), normalized to the β1 subunit, and expressed as a percentage of total cell surface hCTR1 at time 0. The results are presented as the means ± S.D. of three separate experiments. The data were normally distributed. A one-way ANOVA followed by a Games-Howell test was used to determine significant differences between cells to which no medium was added compared with cells treated with medium for 5–30 min subsequent to the addition of 20 μM copper for 10 min. The asterisk denotes statistical significance at $p < 0.05$. The down arrow indicates the addition of 20 μM copper to the cells for 10 min, and the up arrow indicates the removal of copper from the cells.
min of the removal of extracellular copper (Fig. 7B). Because the levels of hCTR1 that were internalized at higher concentrations of copper, were somewhat greater (100 μM) (Fig. 1), we examined whether hCTR1 surface recovery was slower and whether hCTR1 recycled to the plasma membrane as rapidly as at 20 μM copper. We found that hCTR1 took up to 2 h to return to steady state levels at the cell surface after incubation for 10 min with 100 μM copper (data not shown).

Copper-dependent Decrease in Plasma Membrane Levels of Endogenous hCTR1—It was important to determine whether or not endogenous hCTR1 is also internalized in response to elevated copper, as we had shown for the overexpressed protein. We used cell surface biotinylation, the same method used to detect changes in cell surface levels of overexpressed hCTR1 in HEK293 cells, to measure plasma membrane levels of CTR1 in MDCK, HeLa, HepG2, and T47D cells. MDCK cells are a canine renal epithelial cell line, and HepG2, HeLa, and T47D are human cell lines derived from liver, cervical, and breast tissue, respectively. In polarized MDCK cells grown in transwells we observed a decrease in basolateral levels of CTR1 in response to 2.5 and 20 μM copper for 15 min (Fig. 8). In HepG2 and HeLa cells we also saw a decrease in cell surface hCTR1, as shown in Fig. 8, under the same conditions. However, in T47D cells we could detect no change in hCTR1 levels in response to added extracellular copper. These results indicate that endogenous hCTR1 responds in a similar way to the overexpressed protein but that the phenomenon shows cell-specific variation.

DISCUSSION

In the present work we have demonstrated that as well as mediating copper entry into cells, hCTR1 plays a role in the acute regulation of cellular copper acquisition. We have observed a rapid copper-dependent internalization of hCTR1 that is triggered by extracellular copper levels in the micromolar range that results in a decrease in the rate of copper uptake by the cell. When copper is removed from the extracellular medium, intracellular hCTR1 rapidly returns to the plasma membrane. Thus when the cell is confronted by elevated copper levels, the uptake of deleteriously high amounts of copper is reduced by removing the transporter from the surface membrane. When this challenge is removed the transporter rapidly returns to the plasma membrane. Thus the internalization and recycling provides an acute regulatory mechanism for cellular copper homeostasis.

Endogenous and Overexpressed hCTR1—In previous studies in HEK293 cells, confocal imaging failed to see a redistribution of endogenous hCTR1 in response to elevated copper levels (24). It is likely that the combination of low levels of endogenous protein, together with cross-reactivity with an intracellular (38 kDa) protein (17) combined to obscure any copper-dependent relocation. Using confocal imaging, the location of hCTR1 in Caco-2 and HeLa cells was unaltered when exposed to copper levels as high as 400 μM for up to 2 h (14). In our experience it is difficult to obtain hCTR1 antibodies with sufficiently high affinity and specificity that fractional changes in the cellular localization of low levels of endogenous hCTR1 can be confidently demonstrated by imaging. Our present study on overexpressed and endogenous protein using cell surface biotinylation is a more accurate and quantitative assessment of surface hCTR1. Using this technique we observed internalization of overexpressed hCTR1 in response to elevated extracellular copper (2.5–100 μM copper) in HEK293 cells. This result agrees
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with a previous study using this cell system that demonstrated that overexpressed hCTR1 is removed from the cell surface and internalized (23). However, the previous study (23), which reported that hCTR1 was internalized within 10 min, also reported that it was degraded as shown by a decrease in total cellular hCTR1 levels. We have shown that the amount of hCTR1 that disappears from the cell surface (Figs. 1 and 3) can be accounted for by that which we detect in the intracellular compartment (Fig. 5). Using antibodies that detect both the C terminus of hCTR1 and an N terminus attached epitope, we can detect no degradation products of hCTR1 nor a decrease in total cellular hCTR1 levels (Fig. 4). We have been unable to detect degradation of internalized hCTR1 in HEK293 cells within 2 h of copper addition. In our experiments, when copper is removed from the extracellular milieu, the internalization process is reversed, and the transporter recycles to the plasma membrane to mediate renewed copper entry (Fig. 6).

To address the question as to whether copper-induced internalization of overexpressed hCTR1 reflects what also occurs with endogenous protein, we measured the acute response of endogenous CTR1 in MDCK, HeLa, HepG2, and T47D cells. We observed copper-induced internalization of CTR1 in polarized MDCK cells at both 2.5 and 20 μM copper. Our results also confirm the exclusive basolateral location of CTR1 in MDCK cells (15). In HepG2 cells, we detected a significant internalization of hCTR1 in response to extracellular copper. In HeLa cells the change we observed was not at a high level of statistical significance. However, in T47D cells there was no decrease in hCTR1 in response to extracellular copper (Fig. 8). This suggests to us that the response of overexpressed hCTR1 to extracellular copper reflects the response of endogenous hCTR1, but the phenomenon shows cell or tissue specificity. Previous studies reported there was no change in the cellular localization in response to elevated copper (400 μM) in Caco-2 and HeLa cells (14).

Physiological Levels of Copper—We employed a broad range (2.5–100 μM copper) in our study. The reported serum levels of copper are between 5.8 and 53.9 μM (32). The majority of our studies were carried out between 1 and 20 μM within this range. However, there have been earlier studies of hCTR1 responses to extracellular copper that have included the use of copper as high as 100 μM (23) or 400 μM (14). We included the high copper values into the present study to enable a comparison of our results with the earlier work.

Acute Regulation of hCTR1—It was initially puzzling that on exposure to elevated copper, even up to high levels (100 μM), only a fraction of the surface hCTR1 was internalized. Furthermore because this fraction could not be greatly increased by increasing the level of extracellular copper, it seemed to imply that a fraction of the plasma membrane hCTR1 molecules (~40%) might respond differently than the remainder. However, the observation of a low but measurable fraction of internalized hCTR1 in the absence of added extracellular copper, reported here and previously (23), provides an explanation. We consider hCTR1 as being composed of two populations, an intracellular population and a plasma membrane pool. Under very low copper conditions, in cells grown in basal medium containing ~1 μM copper, we could detect most of the hCTR1 at the plasma membrane and a small amount in an internal compartment (–14% of cell surface hCTR1). The distribution between these pools can be thought of as pseudo-equilibrium. When extracellular copper is elevated, the internalization rate is increased, resulting in a greater proportion of hCTR1 molecules being in the internal compartment. When copper is removed, the internalization returns to its original value, and the distribution returns to its resting state. The incomplete removal of hCTR1 from the surface merely represents the balance between the rates of the inward and outward processes.

It should be emphasized that in our experiments we are measuring the distribution of hCTR1 between the plasma membrane and the intracellular compartment. We do not measure the rate constants of internalization and return to the plasma membrane. Our observations could be accounted for by proposing either a copper-dependent increase in the rate of internalization or a copper-dependent reduction in the rate of reinsertion in the plasma membrane. We prefer the former because we believe that the rapid response to extracellular copper most likely acts at the external surface of hCTR1 on the inward rate. Previous studies on internalization pointed out the importance of extracellular methionine residues (Met15 and Met153) in this process (29).

This model suggests that intracellular hCTR1 is in rapid contact (perhaps close proximity) with the plasma membrane. In addition, it may imply that in different cells the resting distribution of internal and surface hCTR1 may differ and that the extent to which internalization may be increased may also vary. Either or both possibilities would allow for the cell-specific differences that have been reported. Our observation that following the internalization of hCTR1, if extracellular copper is removed, hCTR1 returns to the surface provides an important step in an acute regulatory mechanism for copper uptake. In other words, this process can respond rapidly to elevations of extracellular copper and reduce its harmful entry into cells but also returns rapidly to allow for optimal uptake when the threat is removed.

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