

## Copper-stimulated Endocytosis and Degradation of the Human Copper Transporter, hCtr1\*

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**Copper uptake at the plasma membrane and subsequent delivery to copper-dependent enzymes is essential for many cellular processes, including mitochondrial oxidative phosphorylation, free radical detoxification, pigmentation, neurotransmitter synthesis, and iron metabolism. However, intracellular levels of this nutrient must be controlled because it is potentially toxic in excess concentrations. The hCtr1 protein functions in high affinity copper uptake at the plasma membrane of human cells. In this study, we demonstrate that levels of the hCtr1 protein at the plasma membrane of HEK293 cells were reduced when cells were exposed to elevated copper. This decrease in surface hCtr1 levels was associated with an increased rate of endocytosis, and low micromolar concentrations of copper were sufficient to stimulate this process. Inhibitors of clathrin-dependent endocytosis prevented the trafficking of hCtr1 from the plasma membrane, and newly internalized hCtr1 and transferrin were co-localized. Significantly, elevated copper concentrations also resulted in the degradation of the hCtr1 protein. Our findings suggest that hCtr1-mediated copper uptake into mammalian cells is regulated by a post-translational mechanism involving copper-stimulated endocytosis and degradation of the transporter.**

Copper is an essential nutrient for all organisms because it is required by a variety of enzymes that are involved in critical areas of metabolism (1). Because copper is also potentially toxic when it accumulates beyond cellular needs, intracellular levels of this nutrient must be precisely regulated. In the yeast, *Saccharomyces cerevisiae*, yCtr1 and yCtr3, are plasma membrane-associated high affinity copper transporters (2, 3). Recent studies have identified the human and mouse high affinity copper uptake proteins, hCtr1 and mCtr1 (4, 5). Like the yCtr1 protein, the mammalian homologues possess three predicted membrane spanning regions and form oligomeric complexes, possibly to facilitate formation of a membrane pore for copper uptake (6–8). Deletion of the *mCtr1* gene in mice results in embryonic lethality, suggesting mCtr1-mediated copper uptake is essential during mammalian embryogenesis (9, 10). In mouse embryonic cell lines in which both *mCtr1* alleles have

been disrupted, we have recently shown that a myc-tagged human Ctr1 protein functions in copper uptake and delivery to the copper-dependent enzymes cytochrome *c* oxidase, superoxide dismutase, and tyrosinase (11). In the cultured human embryonic kidney cell line, HEK293,<sup>1</sup> the hCtr1-myc protein is predominantly located at the plasma membrane and functions in copper uptake in a time-, temperature-, pH-, and K<sup>+</sup>-dependent manner (8). Recent studies suggest that hCtr1 is predominantly in perinuclear vesicles in some cell lines, whereas in others it is at the plasma membrane (7, 8). The basis for this variability in hCtr1 localization in different cell lines is unknown.

In yeast, the expression of yCtr1 is up-regulated via the Mac1p transcription factor when extracellular copper levels are low (12–14). In addition to this transcriptional regulation of yCtr1 expression, the levels of the protein at the plasma membrane are post-translationally regulated by copper availability. When yeast cells are exposed to low micromolar levels of copper, yCtr1 is stimulated to endocytose from the plasma membrane to cytoplasmic vesicles (15). This could serve to rapidly prevent excessive copper uptake via yCtr1 and reduce the potential for copper toxicity, or alternatively, provide a vesicular route for yCtr1-mediated copper uptake. At higher concentrations of copper (~10 μM) the yCtr1 protein is degraded at the plasma membrane via a mechanism that is independent of endocytosis (15). Despite progress in understanding the biochemical aspects of hCtr1-mediated copper transport in human cells, it is unknown whether the function of hCtr1 is regulated by copper availability. Unlike yeast, there is currently no evidence for copper-regulated transcription of mammalian *Ctr1* (5). Therefore, we tested whether copper levels in the growth media regulate hCtr1 distribution in cultured mammalian cells. Using HEK293 and Chinese hamster ovary (CHO) cell lines transfected with a functional myc-tagged hCtr1 protein, we showed that the level of hCtr1 protein at the plasma membrane was reduced when cells were exposed to elevated copper in the media. The reduced levels of hCtr1 at the plasma membrane were associated with an increased rate of hCtr1 endocytosis, which was specifically inducible by low micromolar copper levels. Higher copper levels resulted in the degradation of hCtr1. Our findings suggest that copper-stimulated endocytosis and degradation of the hCtr1 protein could play a key role in regulating copper entry across the plasma membrane of mammalian cells.

### EXPERIMENTAL PROCEDURES

*Reagents, Cell Lines, and Antibodies*—The human embryonic kidney (HEK293) cell lines expressing either the myc-tagged hCtr1 protein, or

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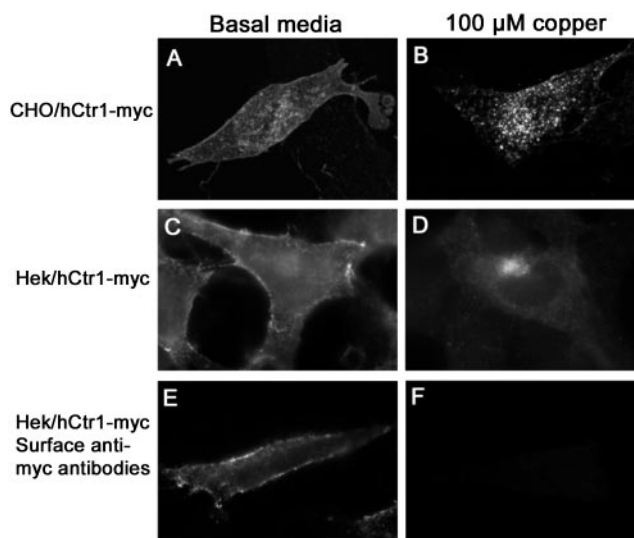
<sup>1</sup> The abbreviations used are: HEK293, human embryonic kidney 293; CHO, Chinese hamster ovary; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; MCD, methyl-β-cyclodextrin.

pcDNA3.1 vector, were isolated by transfection using the LipofectAMINE 2000 reagent (Invitrogen) as previously described (8). The functional c-myc epitope-tagged hCtr1 expression construct in pcDNA3.1(+) vector and pTK-Hyg (Clontech) were cotransfected in HEK293 cells. Hygromycin-resistant cell lines were established by the selection of transfected cells with the supplementation of 100  $\mu\text{g}/\text{ml}$  hygromycin B (Invitrogen) in the culture medium. hCtr1 expression was analyzed by Western blotting using the anti-myc antibody and by measuring stimulation of  $^{64}\text{Cu}$  uptake, as described previously (8). CHO cells were transiently transfected using LipofectAMINE 2000 using the pcDNA3.1 plasmid containing the myc-tagged hCtr1 cDNA as previously described (8). All cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 units/ml penicillin and streptomycin in a 5%  $\text{CO}_2$ , 37 °C incubator. Monoclonal anti-myc antibody, 9E10, streptavidin-horseradish peroxidase (HRP) conjugate, and anti-mouse antibodies conjugated to HRP were purchased from Roche Molecular Biochemicals. Alexa 488 anti-mouse antibodies, transferrin-biotin conjugate, and transferrin-Texas Red were purchased from Molecular Probes. Chlorpromazine, cycloheximide, and methyl- $\beta$ -cyclodextrin were purchased from Sigma.

**Immunofluorescence Microscopy to Assess Surface and Intracellular Pools of hCtr1 Protein**—Cells were grown in 24-well trays for 48 h on sterile glass coverslips. In some experiments, copper was added to the media at the indicated concentrations and times. To detect the total pool of hCtr1-myc protein, cells were washed twice with 1 ml of ice-cold PBS and fixed for 10 min at 25 °C using 4% paraformaldehyde. The cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 min, blocked for 1 h with 1% bovine serum albumin and 3% skim milk in PBS, and then probed with the anti-myc antibodies (10  $\mu\text{g}/\text{ml}$ ) followed by Alexa 488 anti-mouse antibodies (1/1000). To label only the surface pool of hCtr1-myc, the Triton X-100 permeabilization step was omitted and fixed cells were blocked and probed with the anti-myc and Alexa 488 antibodies as described above. The endocytosis of hCtr1-myc was assessed by detecting the uptake of anti-myc antibodies added to the media of Hek/hCtr1-myc cells. Cells were pre-grown for 48 h in basal media and then incubated for either 2 or 5 min in basal- or copper-supplemented media containing 10  $\mu\text{g}/\text{ml}$  anti-myc antibodies at 37 °C. Cells were transferred to ice to prevent further trafficking of hCtr1-myc, washed twice with ice-cold PBS, and the surface-bound antibodies were removed by three washes for 2 min with acidic buffer on ice (100 mM glycine, 20 mM magnesium acetate, 50 mM potassium chloride, pH 2.2). After a further two washes with PBS on ice, cells were fixed, permeabilized, and processed for immunofluorescence as described above. In double labeling endocytosis experiments, 100  $\mu\text{g}/\text{ml}$  Texas Red-conjugated transferrin and 10  $\mu\text{g}/\text{ml}$  anti-myc antibodies were added to the media of cells for 2 min and processed for immunofluorescence microscopy as described above. Confocal immunofluorescence microscopy was performed with a  $\times 60$  oil objective and an IX70 Olympus microscope fitted with a Bioradiance 2000 laser (Bio-Rad).

**Detection of hCtr1 Protein Levels at the Plasma Membrane**—The pool of hCtr1-myc at the plasma membrane was assessed by measuring the levels of anti-myc antibodies bound to the surface of Hek/hCtr1-myc cells. Hek/hCtr1-myc cells were cultured for 48 h in 6-well trays, washed twice with PBS on ice, and fixed for 10 min in 4% paraformaldehyde without subsequent permeabilization steps. Cells were then blocked using 3% skim milk in PBS, and incubated with the anti-myc antibody (1/500) for 30 min at room temperature. Cells were washed five times in PBS to remove unbound antibodies, and then lysed by sonication in SDS buffer solution containing 62 mM Tris-Cl (pH 6.8), 2% SDS, 100 mM dithiothreitol, and protease inhibitor mixture (Roche Molecular Diagnostics). Cell lysates containing the solubilized anti-myc antibodies that were bound to the hCtr1-myc protein at the plasma membrane were separated using 4–20% SDS-PAGE, transferred to nitrocellulose membranes, and the anti-myc antibodies were then detected using anti-mouse HRP antibodies (1/5000) by chemiluminescence (Roche Molecular Biochemicals). Tubulin protein levels were detected on parallel immunoblots using anti-tubulin antibodies (1:40,000; Sigma).

**Assay of hCtr1 Endocytosis**—The endocytosis of hCtr1-myc was determined by measuring the uptake of anti-myc antibodies added to the cultured media of Hek/hCtr1-myc cells. Cells were pre-grown in 6-well trays for 48 h in basal media, and then incubated for the indicated times at 37 °C in basal- or copper-supplemented media containing 10  $\mu\text{g}/\text{ml}$  anti-myc antibodies. Cells were washed twice with PBS on ice, and surface-bound antibodies were removed by three washes in ice-cold acidic buffer (above). Cells were harvested by scraping into ice-cold PBS and pelleted by centrifugation at 1000  $\times g$ . The cell pellets were solubilized in SDS buffer (above), and 20  $\mu\text{g}$  of lysates containing internal-



**FIG. 1. Elevated copper alters the localization of hCtr1.** Immunofluorescence microscopy analysis of hCtr1-myc distribution in CHO and HEK293 cells. CHO cells transiently transfected with the hCtr1-myc expression plasmid (A and B), or HEK293 cells stably expressing myc-tagged hCtr1 protein (Hek/hCtr1-myc) (C–F), were cultured in basal media (A, C, and E) or media containing 100  $\mu\text{M}$  copper for 16 h (B, D, and F). The total pool of hCtr1-myc was detected using anti-myc antibodies after fixing cells with 4% paraformaldehyde and then permeabilizing with 0.1% Triton X-100 prior to detection using anti-myc antibodies (A–D). The surface pool of hCtr1-myc protein is shown for untreated- and copper-treated Hek/hCtr1-myc cells that were fixed with 4% paraformaldehyde without permeabilization (E and F).

ized anti-myc antibodies were separated using 4–20% SDS-PAGE, transferred to nitrocellulose membranes, and detected by chemiluminescence using anti-mouse HRP antibodies, as described above. In some experiments, 100  $\mu\text{g}/\text{ml}$  biotinylated transferrin was added to the media instead of anti-myc antibodies and the internalized transferrin was detected using streptavidin-peroxidase on Western blots.

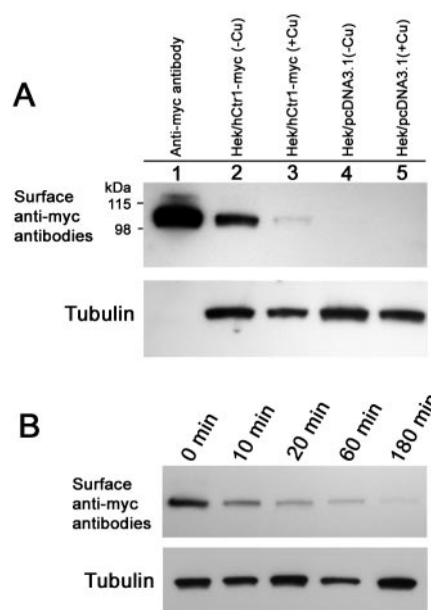
**Immunoblot Analysis of hCtr1 Protein**—Immunoblot detection of hCtr1-myc protein was essentially as described previously (8). Cells cultured in 25-cm<sup>2</sup> flasks were scraped into ice-cold PBS and pelleted by centrifugation. After several washes in ice-cold PBS, the cells were lysed for 20 min on ice in buffer containing 1% Triton X-100, 1 mM EDTA, 100 mM dithiothreitol, and protease inhibitor mixture (Roche Molecular Biochemicals). Samples were centrifuged for 10 min at 16,000  $\times g$  and 20  $\mu\text{g}$  of protein lysates were separated using 4–20% SDS-PAGE, transferred to nitrocellulose membranes, and detected by chemiluminescence using anti-myc antibodies (1:1000) followed by anti-mouse HRP antibodies (1/5000).

## RESULTS

**Elevated Copper Concentrations Reduce hCtr1 Levels at the Plasma Membrane**—This study was aimed at determining whether the levels and distribution of the hCtr1 copper transporter is subject to regulation by copper. We previously engineered a human Ctr1 cDNA with an amino-terminal myc epitope (hCtr1-myc) in the expression plasmid pcDNA 3.1, and demonstrated this tagged protein is a functional copper transporter and located at the plasma membrane in transfected HEK293 cells (8). Our initial studies investigated the localization of hCtr1-myc transiently expressed in CHO cells cultured in growth media containing elevated copper. Immunofluorescence microscopy using the anti-myc antibody revealed strong labeling of hCtr1-myc at the plasma membrane of CHO cells grown in basal media, with some punctate vesicular labeling in the cytoplasm (Fig. 1A). However, in cells exposed to elevated copper there was a striking change in localization to a punctate vesicular distribution throughout the cytoplasm, and a marked reduction in levels of hCtr1-myc at the plasma membrane (Fig. 1B). These observations suggested that the location of hCtr1-myc in CHO cells was altered by elevated copper levels. We

then investigated the effect of copper on the location of hCtr1-myc in the HEK293 cell line, Hek/hCtr1-myc, which stably expresses the hCtr1-myc protein (8). The plasma membrane location for hCtr1-myc in Hek/hCtr1-myc cells grown in basal medium was confirmed (Fig. 1C). However, similar to that observed for CHO cells, when Hek/hCtr1-myc cells were exposed to elevated copper levels the hCtr1-myc protein was localized in a perinuclear distribution, and there was little apparent staining at the cell periphery (Fig. 1D). There was no immunofluorescence signal detected in CHO or HEK293 cells transfected with the pcDNA3.1 vector alone (data not shown). These data suggest that elevated copper concentrations reduced the levels of hCtr1-myc at the plasma membrane in both HEK293 and CHO cells.

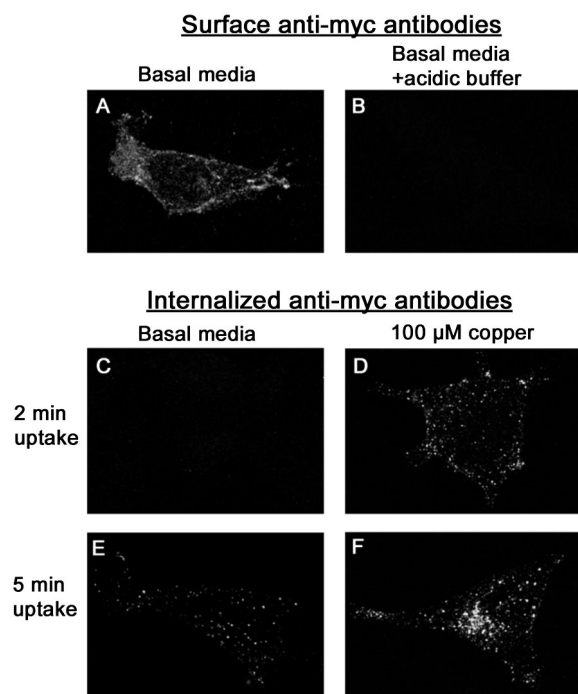
Previous studies have demonstrated that the amino terminus of hCtr1 is extracellular (6, 16), and this topology allowed us to take advantage of the amino-terminal position of the myc epitope tag to directly test whether elevated copper reduces hCtr1 levels at the plasma membrane. Hek/hCtr1-myc cells were grown in basal media or media containing 100  $\mu\text{M}$  copper for 16 h, and immunofluorescence microscopy was carried out with the anti-myc antibodies using intact paraformaldehyde-fixed cells that had not been permeabilized. Strong labeling of hCtr1-myc was observed at the surface of intact Hek/hCtr1-myc cells grown in basal medium (Fig. 1E), however, there was no surface labeling of hCtr1-myc in copper-treated cells (Fig. 1F). The integrity of the plasma membrane was confirmed after fixation by the absence of labeling with antibodies against the intracellular protein, protein-disulfide isomerase (data not shown). As an independent assay, immunoblots were used to assess the reduced levels of hCtr1-myc protein at the plasma membrane of copper-treated Hek/hCtr1-myc cells. This was achieved by measuring the levels of anti-myc antibodies that were recovered from the surface of intact Hek/hCtr1-myc cells after these cells were probed with anti-myc antibodies. Hek/hCtr1-myc cells were grown in basal- or copper-supplemented media, fixed with paraformaldehyde, and the intact cells were then probed with the anti-myc antibodies to label hCtr1-myc protein at the plasma membrane. After extensive washing of cells with PBS to remove unbound antibodies, the cells were lysed with SDS buffer and the solubilized anti-myc antibodies were detected by SDS-PAGE and Western blotting with anti-mouse antibodies conjugated to HRP. As a control, the affinity purified anti-myc antibodies were run on the same gel and detected as a single 100-kDa band (Fig. 2A, lane 1). The 100-kDa band was strongly detected in the sample from Hek/hCtr1-myc cells grown in basal medium, demonstrating abundant levels of anti-myc antibodies on the surface because of the myc epitope of hCtr1 (Fig. 2A, lane 2). Markedly reduced levels of anti-myc antibodies were recovered from the surface of cells grown in elevated copper (Fig. 2A, lane 3). There were no detectable anti-myc antibodies bound to the surface of untreated- or copper-treated HEK293 cells stably transfected with the empty pcDNA3.1 vector (Fig. 2A, lanes 4 and 5). These findings independently confirmed the immunofluorescence microscopy results and suggested that elevated copper reduces the level of hCtr1-myc protein at the plasma membrane. We then explored the kinetics of hCtr1 clearance from the plasma membrane following exposure of Hek/hCtr1-myc cells to elevated copper. A clear reduction in the levels of surface-bound anti-myc antibodies occurred after a 10-min exposure to elevated copper, and decreased over the course of 180 min (Fig. 2B). This decrease in surface hCtr1-myc expression occurred independently of *de novo* protein synthesis, because we observed the same effect in cells pretreated with the translation inhibitor, cycloheximide,



**FIG. 2. Elevated copper reduces surface expression of hCtr1.** A, immunoblot analysis of anti-myc antibodies bound to the surface of Hek/hCtr1-myc cells (lanes 2 and 3) or HEK293 cells stably transfected with pcDNA3.1 vector (lanes 4 and 5) that were cultured for 16 h in basal media (-Cu) or 100  $\mu\text{M}$  copper (+Cu). Cells were fixed using paraformaldehyde, blocked, and then probed with anti-myc antibodies. The surface-bound antibodies were solubilized with SDS buffer, separated using SDS-PAGE, and detected by immunoblotting with anti-mouse antibodies conjugated to HRP as described under "Experimental Procedures." Purified anti-myc 9E10 antibodies were run as a control (lane 1), and tubulin protein was detected to indicate protein loading. B, immunoblot analysis of anti-myc antibodies bound to the surface of Hek/hCtr1-myc cells after the cells were cultured in media containing 100  $\mu\text{M}$  copper for the indicated times, as per A and under "Experimental Procedures."

for 30 min followed by exposure to media containing both copper and cycloheximide (data not shown).

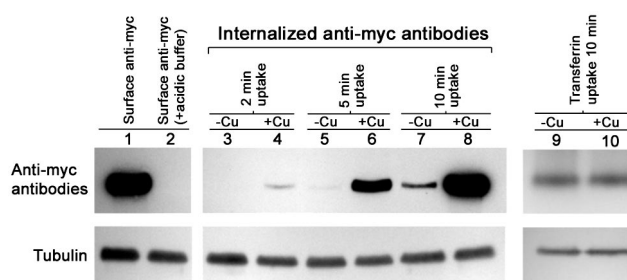
**Copper-stimulated Endocytosis of hCtr1**—The rapidity with which surface levels of hCtr1 were reduced in response to elevated copper prompted us to test whether this is accomplished through copper-stimulated endocytosis of hCtr1. We exploited the extracellular location of the myc epitope of hCtr1-myc to assess endocytosis of the protein. Using the same strategy previously employed to assess endocytosis of the Menkes disease copper transporter, ATP7A (17), we surmised that if hCtr1-myc endocytosed from the plasma membrane, anti-myc antibodies added to the growth media would bind to hCtr1-myc and be internalized by Hek/hCtr1-myc cells. Initially, immunofluorescence microscopy was used to demonstrate that the surface pool of hCtr1-myc could be labeled by the anti-myc antibodies added to the media of living Hek/hCtr1-myc cells that were cooled on ice to inhibit endocytosis (Fig. 3A). There was no labeling by HEK293 cells transfected with the pcDNA3.1 vector (data not shown), indicating that the anti-myc antibodies were specifically bound to the extracellular myc epitope of the hCtr1-myc protein in Hek/hCtr1-myc cells. We found that several washes with cold acidic buffer removed the surface-bound antibody (Fig. 3B). This washing step was important in establishing conditions that would remove surface antibodies and permit the visualization of internalized antibodies in subsequent endocytosis experiments. To test the effect of copper on the endocytosis of hCtr1-myc, cells were pre-grown for 48 h in basal media and then incubated for 2 or 5 min in basal or elevated copper media containing anti-myc antibodies to allow uptake of the antibodies via hCtr1-myc endocytosis. After these antibody uptake time periods were completed, cells were rapidly cooled



**FIG. 3. Elevated copper stimulates endocytosis of hCtr1-myc.** The hCtr1-myc protein at the plasma membrane of living Hek/hCtr1-myc cells was labeled with anti-myc antibodies on ice. Cells were then washed with either PBS (A) or acidic buffer (B) prior to fixation, permeabilization, and detection by immunofluorescence microscopy. Note the removal of surface-associated antibodies with acidic buffer. C–F, assessment of hCtr1-myc endocytosis through the uptake of extracellular anti-myc antibodies. Hek/hCtr1-myc cells pre-grown in basal media were exposed for 2 (C and D) or 5 min (E and F) to anti-myc antibodies in basal media (C and E) or media containing 100  $\mu\text{M}$  copper (D and F). Cells were then washed with acidic buffer to remove surface antibodies and the intracellular antibodies were then detected by immunofluorescence microscopy.

on ice and the surface-bound anti-myc antibodies were removed by washing cells with ice-cold acidic buffer. The protected internalized antibodies were then detected by confocal immunofluorescence microscopy. After 2 min there was no apparent uptake of anti-myc antibodies by cells in basal media (Fig. 3C). However, after 2 min in elevated copper, anti-myc antibodies were detected in vesicular compartments close to the cell periphery (Fig. 3D). Interestingly, by 5 min in basal media, the anti-myc antibodies had accumulated to low levels in peripheral compartments (Fig. 3E). This indicated that endocytosis of hCtr1-myc had occurred in the absence of copper supplementation. However, after 5 min in elevated copper the staining of intracellular anti-myc antibodies was more intense than in basal media and there was a substantial concentration in the perinuclear region (Fig. 3F). There was no internalization of anti-myc antibodies by untreated or copper-treated HEK293 cells transfected with the pcDNA3.1 vector (data not shown), suggesting that the uptake of anti-myc antibodies in the Hek/hCtr1-myc cells occurred through specific binding to hCtr1-myc protein. These data suggest that the hCtr1-myc protein undergoes endocytosis in basal media, and that elevated copper stimulates this process.

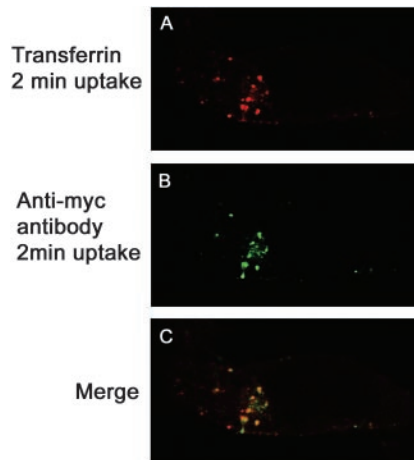
To more quantitatively demonstrate copper-induced endocytosis of hCtr1-myc, we measured levels of internalized anti-myc antibodies by immunoblotting experiments. As we had done earlier for the immunofluorescence experiments, we initially confirmed that washing cells with acidic buffer removes surface-bound anti-myc antibodies. Hek/hCtr1-myc cells were pre-grown for 48 h in basal media, cooled on ice, and then incubated for 10 min with the anti-myc antibodies to label hCtr1-myc



**FIG. 4. Time course analysis of copper-stimulated endocytosis of hCtr1-myc by immunoblot detection.** Hek/hCtr1-myc cells were labeled on ice with anti-myc antibodies, washed with either PBS (lane 1) or acidic buffer (lane 2), and the antibodies were detected using immunoblotting experiments. This result demonstrated the removal of surface-bound anti-myc antibodies by the acidic buffer washes. hCtr1-myc endocytosis was determined by assessing the uptake of anti-myc antibodies at 37  $^{\circ}\text{C}$ . Cells were incubated in the presence of 10  $\mu\text{g}/\text{ml}$  anti-myc antibodies added to basal media (–Cu) or media containing 100  $\mu\text{M}$  copper (+Cu) for the indicated times. Surface antibodies were removed by washing cells in cold acidic buffer and intracellular antibodies were then detected by SDS-PAGE and immunoblotting using anti-mouse antibodies conjugated to HRP (lanes 3–8). Note the reduced uptake of antibodies from basal media relative to elevated copper. In parallel experiments, the uptake of biotinylated transferrin over 10 min from basal media (–Cu) or 100  $\mu\text{M}$  copper media (+Cu) was assessed by immunoblotting (lanes 9 and 10). Tubulin levels are shown in the lower panel to indicate protein loading for all samples.

protein at the plasma membrane. Cells were either washed with PBS or acidic buffer on ice, lysed in SDS buffer, and the anti-myc antibodies were detected on Western blots. The 100-kDa band corresponding to the anti-myc antibody was detected in the samples derived from PBS-washed cells (Fig. 4, lane 1), however, this was absent from cells that were washed with acidic buffer (Fig. 4, lane 2). This indicated that surface-bound anti-myc antibodies were removed by this acidic buffer treatment. To explore the effect of elevated copper on the endocytosis of hCtr1-myc, the Hek/hCtr1-myc cells were pre-grown for 48 h in basal media and then incubated in either basal or 100  $\mu\text{M}$  copper media containing the anti-myc antibodies. Cells were transferred to ice to prevent further trafficking, washed extensively with acidic buffer to remove surface-bound anti-myc antibodies, and cell lysates were prepared to allow intracellular anti-myc antibodies to be detected on immunoblots. After a 2-min uptake period, internalized anti-myc antibodies were detected in copper-treated cells (Fig. 4, lane 4), however, no signal was detected in cells exposed to basal media (Fig. 4, lane 3). However, after 5 min of uptake the anti-myc antibodies were weakly detected in cells exposed to basal media (Fig. 4, lane 5), but the levels were 20-fold lower than in the copper-treated cells, as determined by densitometry measurements of band intensity (Fig. 4, lane 6). This elevated uptake of anti-myc antibodies in copper-treated cells relative to untreated cells was also apparent at the 10-min time point (Fig. 4, lanes 7 and 8). As we found earlier in the immunofluorescence studies, there was no internalization of anti-myc antibodies in HEK293 cells stably transfected with the pcDNA3.1 vector alone (data not shown). To address the specificity of copper-induced hCtr1-myc endocytosis, the effects of copper treatment on the uptake of transferrin was evaluated in Hek/hCtr1-myc cells. Elevated copper did not alter the uptake of transferrin (Fig. 4, lanes 9 and 10), indicating that the increased uptake of anti-myc antibodies in copper-treated cells was not because of a general increase in endocytosis from the plasma membrane. These data suggest that elevated copper specifically stimulates the endocytosis of hCtr1-myc from the plasma membrane, and are consistent with earlier results showing a copper-induced decrease in surface levels of hCtr1.

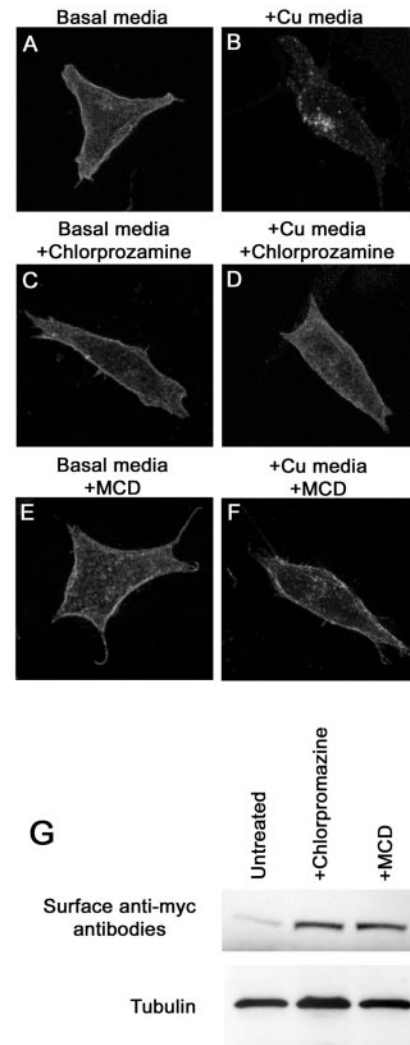
*Co-localization of Newly Internalized hCtr1-myc and Trans-*



**FIG. 5. Newly internalized hCtr1-myc and transferrin are co-localized.** Hek/hCtr1-myc cells were incubated for 2 min in media containing 100  $\mu$ M copper, the anti-myc antibodies, and transferrin conjugated to the Texas Red fluorophore. Cells were then washed with acidic buffer to remove antibodies and transferrin from the plasma membrane, fixed, permeabilized, and intracellular antibodies were detected with Alexa 488 anti-mouse antibodies. Cytoplasmic vesicles containing internalized transferrin (A, red), and anti-myc antibodies (B, green), were extensively co-localized as indicated by yellow compartments in the merged image (C).

*ferrin*—To begin to understand the pathway of hCtr1-myc endocytosis, we determined if newly internalized anti-myc antibodies co-localized with transferrin, a marker of early endosomes originating from clathrin-mediated endocytosis (18). Hek/hCtr1-myc cells were exposed to elevated copper media containing both the anti-myc antibodies and human transferrin conjugated to the Texas Red fluorophore. After a 2-min incubation at 37 °C, the cells were washed with cold acidic buffer to remove any anti-myc antibodies and transferrin bound to the cell surface. Cells were then fixed, permeabilized, and the internal anti-myc antibodies were detected with anti-mouse Alexa 488 antibodies. The internalized transferrin (Fig. 5A, red) and anti-myc antibodies (Fig. 5B, green) were co-localized within vesicular compartments, as shown by the predominantly yellow labeling in the overlay image (Fig. 5C). These findings suggested that newly internalized hCtr1-myc and transferrin co-localize with early endosomes.

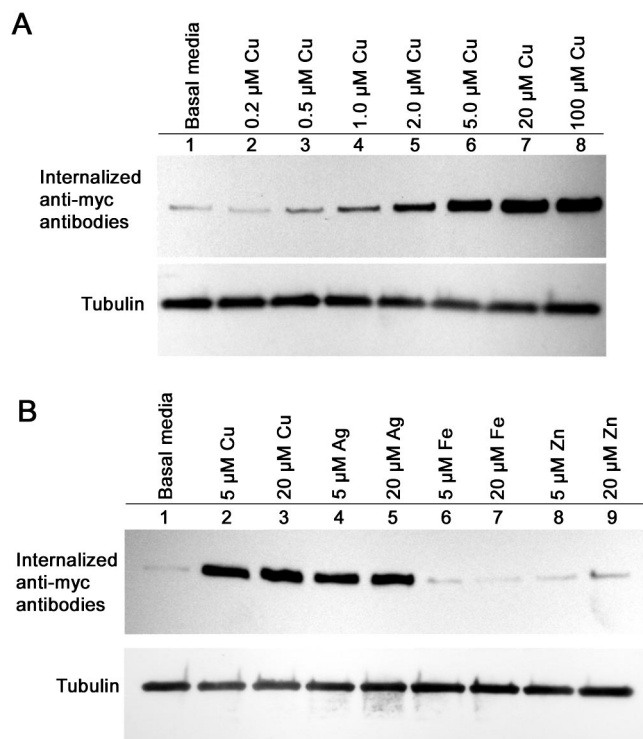
**Copper-induced Internalization of hCtr1-myc Is Clathrin-dependent**—Because transferrin is internalized via clathrin-mediated endocytosis, the finding that newly internalized transferrin and hCtr1-myc were co-localized prompted us to test whether the internalization of hCtr1-myc was clathrin-dependent. Chlorpromazine and methyl- $\beta$ -cyclodextrin (MCD) have been used extensively to inhibit endocytosis of surface proteins. Chlorpromazine is a cationic amphiphilic drug that inhibits the assembly of the clathrin adaptor protein, AP2, on clathrin-coated pits (19), whereas MCD is a more general inhibitor of endocytosis through its ability to extract cholesterol from the plasma membrane and inhibit formation of clathrin-coated pits and caveolae (20). Hek/hCtr1-myc cells were incubated for 20 min with either chlorpromazine or MCD, prior to a 2-h incubation in the presence of the inhibitor plus elevated copper. Immunofluorescence microscopy was then used to assess the intracellular location of hCtr1-myc protein. The expected internalization of hCtr1-myc protein to cytoplasmic vesicles in copper-treated cells was observed in the absence of endocytosis inhibitors (Fig. 6B), as shown earlier (Fig. 1D). Chlorpromazine and MCD treatments did not alter the plasma membrane location of hCtr1-myc in cells in basal media (Fig. 6, C and E). However, the copper-induced internalization of hCtr1-myc protein from the plasma membrane was inhibited in cells treated



**FIG. 6. Chlorpromazine and MCD inhibit copper-induced hCtr1 endocytosis and increase surface levels of hCtr1 in basal media.** A–F, Hek/hCtr1-myc cells were pretreated for 20 min with chlorpromazine (5  $\mu$ g/ml) or MCD (10 mM), and then incubated for 2 h in either basal media containing the endocytic inhibitor or 100  $\mu$ M copper (+Cu) containing the endocytic inhibitor. Control Hek/hCtr1-myc cells were incubated for 2 h in basal media (A) or 100  $\mu$ M copper (B) without endocytosis inhibitors. The location of hCtr1-myc was assessed by immunofluorescence microscopy using anti-myc antibodies. G, analysis of surface levels of hCtr1-myc protein in Hek/hCtr1-myc cells treated with chlorpromazine or MCD. Hek/hCtr1-myc cells were incubated for 2 h in basal media (lane 1), 5  $\mu$ g/ml chlorpromazine (lane 2), or 10 mM MCD (lane 3). Cells were fixed using paraformaldehyde, blocked, and then probed with anti-myc antibodies. The surface-bound antibodies were solubilized with SDS buffer, separated using SDS-PAGE, and detected by immunoblotting with anti-mouse antibodies conjugated to HRP.

with chlorpromazine or MCD (Fig. 6, D and F). In control experiments, the uptake of transferrin was inhibited by both chlorpromazine and MCD (data not shown). These observations suggest that hCtr1 is internalized by clathrin-dependent endocytosis.

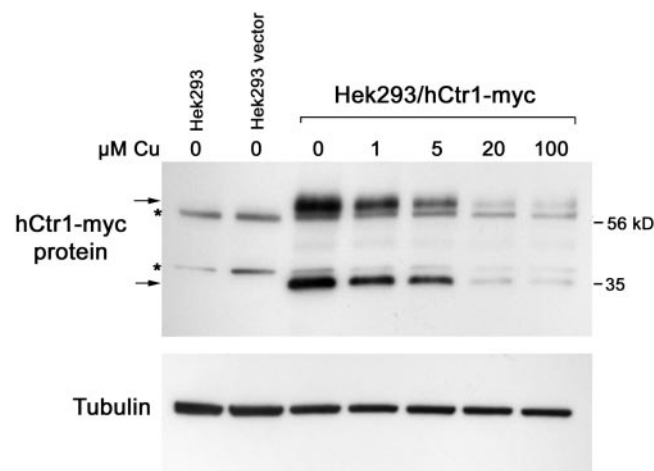
Our earlier finding that anti-myc antibodies were internalized by hCtr1-myc cells in basal media suggested that the hCtr1-myc protein may constitutively cycle via the plasma membrane and intracellular compartments in the absence of elevated copper. To further explore whether hCtr1-myc constantly cycles via the plasma membrane, we tested whether endocytosis inhibitors would result in elevated levels of hCtr1-myc protein at the plasma membrane. To test this hypothesis, we assessed the surface levels of hCtr1-myc protein after incu-



**FIG. 7. Sensitivity and metal specificity of copper-induced hCtr1 endocytosis.** *A*, immunoblots were used to detect internal levels of anti-myc antibodies endocytosed by Hek/hCtr1-myc cells after 10 min in either basal media (*lane 1*) or media containing increased copper concentrations (*lanes 2–8*). Tubulin levels are shown in the *lower panel* to indicate protein loading for all samples. *B*, immunoblots were used to detect intracellular anti-myc antibodies endocytosed by Hek/hCtr1-myc cells after 10 min in either basal media (*lane 1*) or media containing the indicated concentrations of copper (*Cu*) (*lanes 2 and 3*), silver (*Ag*) (*lanes 4 and 5*), iron (*Fe*) (*lanes 6 and 7*), and zinc (*Zn*) (*lanes 8 and 9*). Tubulin levels are shown to indicate protein loading of samples.

bating hCtr1-myc cells in basal media containing chlorpromazine or MCD. hCtr1-myc cells were incubated for 2 h at 37 °C in basal media containing either chlorpromazine or MCD, and surface levels of the hCtr1-myc protein were determined by assessing the recovery of surface-bound anti-myc antibodies from the surface of intact cells. As shown in Fig. 6G, the surface levels of hCtr1-myc protein were elevated in cells treated with chlorpromazine and MCD, relative to untreated cells. These data suggest that although the hCtr1-myc protein has a steady-state localization at the plasma membrane, the protein cycles via intracellular compartments in the absence of elevated copper.

**Sensitivity and Metal Specificity of Copper-induced hCtr1 Endocytosis**—To further characterize the process of copper-stimulated hCtr1-myc endocytosis, we determined the sensitivity of this response. Hek/hCtr1-myc cells were pre-grown in basal media for 48 h, and the uptake of anti-myc antibodies was assessed after 10 min in media containing a range of copper levels. Copper levels as low as 0.5–1.0  $\mu\text{M}$  stimulated the increased internalization of anti-myc antibodies compared with basal media (Fig. 7A, *lanes 3 and 4*), and this progressively increased until saturation was between 5 and 20  $\mu\text{M}$  copper (Fig. 7A, *lanes 6 and 7*). To test metal specificity, the uptake of anti-myc antibodies was investigated in media containing 5 or 20  $\mu\text{M}$   $\text{AgNO}_3$ ,  $\text{FeCl}_2$ , or  $\text{ZnCl}_2$  (Fig. 7B). The uptake of anti-myc antibodies was stimulated by 5 and 20  $\mu\text{M}$  silver ions with similar efficiency to equimolar levels of copper, whereas iron or zinc had relatively little, if any, effect on uptake. Therefore, the stimulation of hCtr1-myc endocytosis is sensitive to low copper concentrations and is somewhat specific for this metal ion.



**FIG. 8. Elevated copper decreases levels of hCtr1-myc protein.** Western blot analysis of steady-state levels of hCtr1-myc in Hek/hCtr1-myc cells after exposure to elevated copper. HEK293, HEK293 cells stably expressing the vector pcDNA 3.1, or Hek/hCtr1-myc cells were pretreated for 20 min with cycloheximide to inhibit protein translation. Hek293/hCtr1-myc cells were then exposed for 2 h to media containing the indicated copper concentrations. Total protein extracts were prepared from cells, separated by SDS-PAGE, transferred to nitrocellulose membranes, and hCtr1-myc protein detected using the affinity purified anti-myc antibody (1:3000) followed by anti-mouse secondary antibodies conjugated to HRP (1:5000). *Arrows* indicate the expected position of monomeric and dimeric forms of hCtr1-myc protein present only in the hCtr1-myc-transfected cells. Note the striking reduction in levels of hCtr1-myc protein in Hek/hCtr1-myc cells exposed to 20 and 100  $\mu\text{M}$  copper levels. *Asterisks* indicate nonspecific cross-reacting proteins that are present in both untransfected and transfected HEK293 cells. Tubulin protein levels in each sample are shown in the *lower panel* to indicate protein loading.

**Copper-induced hCtr1 Degradation**—To further explore the role of copper in regulating the function of hCtr1, we investigated whether levels of the protein were altered in response to elevated copper in the growth media. Hek/hCtr1-myc cells were pre-grown in basal media for 48 h and pretreated for 20 min with 100  $\mu\text{g/ml}$  cycloheximide to inhibit new protein synthesis. Copper was then added to the cycloheximide-containing media at a range of concentrations for 2 h prior to immunoblot analysis of steady-state hCtr1-myc protein levels using anti-myc antibodies (Fig. 8). We and others have previously shown that the hCtr1 protein migrates as a 30–35-kDa monomer and 60–70-kDa dimer by SDS-PAGE (7, 8). These expected sizes for the hCtr1-myc protein were observed in Hek/hCtr1-myc cells after probing Western blots with the anti-myc antibody (Fig. 8, *lane 3, arrows*). Both the 35- and 70-kDa forms of the hCtr1-myc proteins were absent in nontransfected HEK293 cells and HEK293 cells transfected with the expression vector, pcDNA3.1 (Fig. 8, *lanes 1 and 2*). Despite the presence of two weakly cross-reacting proteins endogenous within HEK293 cells (Fig. 8, *asterisks*), the effect of copper on the levels of hCtr1-myc protein was readily detected using the anti-myc antibody. A substantial decrease in levels of hCtr1-myc protein was observed in media containing 1–5  $\mu\text{M}$  copper (Fig. 8, *lanes 4 and 5*), which was further exacerbated at copper concentrations between 5 and 20  $\mu\text{M}$ . Both the 35- and 70-kDa forms of the hCtr1-myc protein (Fig. 8, *lanes 5 and 6*) were decreased by copper treatment. Together with our earlier data, these findings suggest that copper triggers both the endocytosis and degradation of the hCtr1 protein.

#### DISCUSSION

Copper homeostasis is controlled via a number of finely tuned mechanisms through uptake, distribution, and excretion. As a continuation of our previous studies defining the

biochemical and physiological function of hCtr1 in copper uptake, we explored whether hCtr1 levels at the plasma membrane are altered in response to changes in copper concentration. Analysis of hCtr1-myc expressed in CHO and HEK293 cell lines demonstrated that the protein was located at the plasma membrane. Significantly, the surface expression of hCtr1-myc was decreased when CHO and HEK293 cells were exposed to elevated copper in the growth media, although the extent of this decrease was more pronounced in HEK293 cells (Fig. 1). Further analysis using nonpermeabilized Hek/hCtr1-myc cells demonstrated reduced levels of hCtr1-myc at the plasma membrane after a 10-min exposure to 100  $\mu\text{M}$  copper. This observation led us to ask whether copper decreases the surface expression of hCtr1-myc by stimulating hCtr1-myc endocytosis. The extracellular orientation of the myc epitope in hCtr1-myc allowed us to assess endocytosis of the protein by measuring the uptake of anti-myc antibodies from the culture media by Hek293/hCtr1-myc cells. Using both confocal immunofluorescence microscopy and immunoblots, a time-dependent uptake of anti-myc antibodies was observed in both untreated and copper-treated cells. However, the rate of antibody internalization in copper-treated cells was markedly elevated relative to untreated cells. Collectively, these data suggest that elevated copper reduces the levels of hCtr1-myc at the plasma membrane, and that this is associated with increased endocytosis of the protein. The finding that silver also strongly stimulated the endocytosis of hCtr1-myc was significant, as it suggested that monovalent copper, rather than the divalent ion, is the primary substrate for this process. It is also noteworthy that Cu(I) is the primary substrate for copper uptake via hCtr1 (8). The inhibitors of clathrin-mediated endocytosis, chlorpromazine and methyl- $\beta$ -cyclodextrin, prevented the copper-induced relocalization of hCtr1-myc protein from the plasma membrane to cytoplasmic vesicles. Moreover, newly internalized anti-myc antibodies and transferrin were co-localized. Taken together, these data support the notion that hCtr1 enters cells via clathrin-mediated endocytosis and is trafficked to early endosomes. The ability of the hCtr1-myc protein to internalize anti-myc antibodies from basal media suggested the protein was endocytosed in the absence of elevated copper. Moreover, the increased levels of hCtr1-myc protein at the plasma membrane of cells exposed to endocytosis inhibitors provided further evidence supporting the notion that hCtr1 is constantly endocytosed in basal media.

The reduced levels of hCtr1-myc protein at the plasma membrane following copper-stimulated endocytosis is consistent with a homeostatic control mechanism. Under low copper conditions the abundant expression of hCtr1 at the plasma membrane would permit high affinity uptake of copper. However, when extracellular copper concentrations are elevated, the clearance of hCtr1 from the plasma membrane would prevent excessive copper uptake and potential copper toxicity. A notable finding of our study was that elevated copper levels also resulted in the degradation of the hCtr1 protein. The degradation of the protein would provide an additional means to down-regulate copper uptake when copper is present at elevated concentrations, and may also serve to prevent recycling of internalized hCtr1 protein to the plasma membrane. Our findings, together with previous findings that the yeast Ctr1 protein also undergoes copper-stimulated endocytosis and degradation, reveal a remarkably conserved mechanism for regulating copper uptake by eukaryotic cells that probably originated early during evolution.

A significant finding of our study was that copper supplementation of  $\sim 0.5$ – $1.0$   $\mu\text{M}$  in the culture media was sufficient to increase the endocytosis of hCtr1-myc from the plasma mem-

brane. Interestingly, copper uptake via hCtr1-myc in the Hek/hCtr1-myc cell line has a  $K_m$  of  $1.71 \pm 0.39$   $\mu\text{M}$  (8). Hence, the concentrations of copper that stimulate copper uptake via hCtr1 are similar to copper levels that enhance its endocytosis. This observation raises the question of how hCtr1 mediates the uptake of copper into cells. Does copper pass through the lipid bilayer of the plasma membrane as a transport substrate of hCtr1, or as a ligand that enters the cell via endocytosis of a copper-hCtr1 complex? One possibility is that the binding of copper to an extracellular region of hCtr1 protein may trigger endocytosis of the copper-hCtr1 complex to an intracellular compartment from which copper is subsequently transported into the cytoplasm by hCtr1 or an alternative transporter. This endocytic model of copper uptake is reminiscent of the uptake of transferrin-bound iron by the transferrin receptor (21). The observation that hCtr1 undergoes endocytosis in basal media is consistent with an endocytic model of copper uptake. Alternatively, copper may enter the cell via two separate hCtr1-dependent processes: the first via direct hCtr1-mediated transport of copper across the plasma membrane, and the second via endocytosis of the copper-hCtr1 complex. This latter mechanism involving endocytosis may function to supply copper to specific intracellular organelles, either for the purposes of copper storage or for specific metabolic processes.

The copper-stimulated endocytosis of hCtr1 is reminiscent of copper-stimulated endocytosis that has been reported for the mouse and chicken homologues of the prion protein (22). Unlike prion protein endocytosis, which is stimulated by high copper levels ( $>100$   $\mu\text{M}$ ), we observed increased hCtr1 endocytosis at low micromolar copper levels. Although this difference in sensitivity clearly distinguishes copper-induced endocytosis of hCtr1 from that of the prion protein, it remains to be shown whether a common copper-responsive mechanism underlies the endocytosis of both proteins. It will be important to establish whether the copper-stimulated endocytosis of hCtr1 occurs in all cell types that express the protein. The localization of the hCtr1 protein appears to be cell type specific. In HeLa, H441, A549, and HepG2 cell lines, hCtr1 is predominantly located in perinuclear vesicles, however, the protein is localized at the plasma membrane in CaCo2, Ht29, and HEK293 cells (7, 8). This variability in hCtr1 localization may be because of cell type differences in the rates of hCtr1 trafficking between the cell surface and intracellular compartments. Indeed, the recent finding that an inhibitor of endocytosis causes the vesicular pool of hCtr1 to shift to a plasma membrane distribution in HeLa cells suggests that this intracellular pool of hCtr1 cycles via the plasma membrane (7). However, the same study found no evidence that elevated copper affects the steady state location of hCtr1 in CaCo2 or HeLa cells (7), the reasons for which are currently unclear. It is conceivable that copper-stimulated endocytosis of hCtr1 only occurs in certain cell types. Alternatively, elevated copper may not alter the steady-state location of hCtr1 in some cell types because only a small fraction of the total pool of hCtr1 traffics via the plasma membrane, or that different cell types have distinct regulatory pools of copper available to stimulate hCtr1 trafficking.

In summary, we have identified a post-translational mechanism for regulating high-affinity copper uptake in mammalian cells involving copper-stimulated endocytosis and degradation of the hCtr1 copper transporter. We are currently exploring the mechanisms involved in sensing copper, the sorting signals involved in triggering hCtr1 endocytosis, and the vesicular compartments through which hCtr1 traffics. The discovery of copper-stimulated endocytosis of hCtr1 broadens our understanding of how cytoplasmic copper levels are regulated in mammalian cells. Our findings, together with previous studies,

which show that copper triggers the relocation of the Menkes and Wilson copper exporters from the *trans*-Golgi network (23, 24), suggest that copper-induced trafficking regulates the function of all three known high affinity mammalian copper transporters. Understanding how the trafficking of these transporters is coordinated to maintain copper homeostasis will be an important goal of future studies.

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