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Identification of Methionine-rich Clusters That Regulate Copper-stimulated Endocytosis of the Human Ctr1 Copper Transporter*

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Copper uptake and subsequent delivery to copper-dependent enzymes are essential for many cellular processes. However, the intracellular levels of this nutrient must be controlled because of its potential toxicity. The hCtr1 protein functions in high affinity copper uptake at the plasma membrane of human cells. Recent studies have shown that elevated copper stimulates the endocytosis and degradation of the hCtr1 protein, and this response is likely an important homeostatic mechanism that prevents the overaccumulation of copper. The domains of hCtr1 involved in copper-stimulated endocytosis and degradation are unknown. In this study we examined the importance of potential copper-binding sequences in the extracellular domain and a conserved transmembrane ^{150}MXXXM^{154} motif for copper-stimulated endocytosis and degradation of hCtr1. The endocytic response of hCtr1 to low copper concentrations required an amino-terminal methionine cluster (^{40}MMMMPM^{45}) closest to the transmembrane region. However, this cluster was not required for the endocytic response to higher copper levels, suggesting this motif may function as a high affinity copper-sensing domain. Moreover, the transmembrane ^{150}MXXXM^{154} motif was absolutely required for copper-stimulated endocytosis and degradation of hCtr1 even under high copper concentrations. Together with previous studies demonstrating a role for these motifs in high affinity copper transport activity, our findings suggest common biochemical mechanisms regulate both transport and trafficking functions of hCtr1.

Copper is an essential trace element required for several enzymes with critical roles in metabolism, including cytochrome *c* oxidase, lysyl oxidase, dopamine B hydroxylase, superoxide dismutase, and ferroxidases (1). The ability of copper to exist in two redox states, Cu^+ and Cu^{2+}, enables copper-containing enzymes to catalyze electron transfer reactions. However, these same redox properties of copper also make it toxic when present in elevated concentrations within cells. Excess copper can generate the formation of hydroxyl radicals that damage lipids, proteins, and nucleic acids. This paradox of an essential yet toxic nutrient has resulted in the evolution of sophisticated homeostatic mechanisms that maintain an essential supply and controlled distribution of copper while preventing its overaccumulation. Menkes disease and Wilson disease provide genetic evidence of the importance of maintaining copper balance in humans. The Menkes and Wilson disease genes, *ATP7A* and *ATP7B*, respectively, encode two closely related copper-transporting P-type ATPases that are located in the trans-Golgi network (2–4). Excess copper concentrations stimulate the trafficking of the ATP7A protein to the plasma membrane where it effluxes copper (2), whereas ATP7B, which is mainly expressed in the liver, relocates to post-Golgi vesicles where the transporter is thought to compartmentalize copper prior to its excretion (4, 5). The copper-stimulated trafficking of ATP7A/B proteins is an important post-translational mechanism for regulating copper export, and recent studies indicate that copper-responsive trafficking also regulates mammalian copper uptake (6).

Studies in the yeast *Saccharomyces cerevisiae* identified the first high affinity copper uptake proteins, yCtr1[1] and yCtr3 (7, 8). These proteins were the founding members of a widely conserved family of Ctr proteins identified in fungi, plants, insects, and mammals. Yeast mutants lacking yCtr1 and yCtr3 show several phenotypes consistent with a copper deficiency (7–9). Analysis of the human hCtr1 and mouse mCtr1 proteins in mammalian cells indicates these proteins function as copper importers (10, 11). The deletion of mCtr1 in mice results in a severe embryonic lethal phenotype, suggesting that high affinity copper uptake via this protein is critical for normal mammalian development (12, 13). Members of the Ctr family are integral membrane proteins with three membrane-spanning helices, and studies on yCtr1 and hCtr1 indicate that these proteins assemble as multimers (11, 14–16).

Another conserved feature of the Ctr1 protein family is the existence of methionine-rich sequences in the extracellular amino-terminal region (1, 16). These sequences, referred to as "Mets" motifs, have the consensus MXXMXM, and the hCtr1 protein possesses two such motifs. The similarity of Mets motifs to copper-binding domains of the bacterial copper transporters, CopB and CutE, provided initial indications that these sequences may bind copper (8). Recent studies of yCtr1 and hCtr1 proteins have revealed that the Mets motifs are essential for normal copper uptake when extracellular levels are low,

[1] The abbreviations used are: yCtr1 and yCtr3, yeast (*Saccharomyces cerevisiae*) Ctr1 and Ctr3, respectively; mCtr1, mouse Ctr1; hCtr1, human Ctr1; HEK, human embryonic kidney; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; Wt-hCtr1myc, wild type myc-tagged hCtr1.

suggesting that these sequences primarily function to scavenge copper when the metal is scarce (16). All Ctr1 family members also possess an M*XXX*M motif, which is predicted to reside within the second transmembrane domain and is essential for copper transport activity of yCtr1 and hCtr1 (16).

The hCtr1 and yCtr1 proteins, like the ATP7A/B copper exporters, are post-translationally regulated by copper availability. Elevated copper stimulates the rapid endocytosis and degradation of yCtr1 and hCtr1 (6, 17). This process may function to attenuate copper uptake and prevent overaccumulation of this potentially toxic metal. However, the domains of Ctr1 proteins involved in sensing copper and stimulating endocytosis are unknown. In this study, we examined the role of the conserved extracellular Mets motifs, two histidine-rich regions, and the ^{150}M*XXX*M^{154} sequence in regulating the copper-responsive endocytosis and degradation of hCtr1. The Mets motif closest to the first transmembrane region was required to stimulate endocytosis in response to low micromolar copper levels but not elevated concentrations. Moreover, mutation of either methionine of the ^{150}M*XXX*M^{154} motif completely abolished copper-responsive endocytosis and degradation of hCtr1 even under high copper concentrations. These findings suggest a model whereby the endocytic response of hCtr1 to copper may require the protein to be a functional copper transporter.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Lines, and Antibodies—The hCtr1 mutations in this study were either introduced previously into the *hCtr1* cDNA (16) or generated by PCR mutagenesis and subcloned into the pcDNA3.1(+) mammalian expression plasmid. DNA sequencing was used to verify each mutation. The human embryonic kidney cell line HEK293 was cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under 5% CO_2. This basal medium contains ~1 μM copper. Cells were transfected with the pcDNA3.1 empty vector or the same vector harboring the wild type or mutant alleles of hCtr1 using the LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. After selection for 10 days with 500 μg/ml G418, the expression of the wild type hCtr1 and each mutant protein was confirmed by immunofluorescence microscopy and Western blotting (described below) using the 9E10 monoclonal affinity-purified anti-myc antibody (Roche Applied Science). In specific experiments, *de novo* protein synthesis was inhibited by pretreating cells for 30 min with 100 μg/ml cycloheximide (ICN), which was subsequently maintained throughout copper treatments.

Immunofluorescence Microscopy—Cells were grown overnight on sterile glass coverslips coated with poly(L-lysine) (Sigma), washed twice with 1 ml of ice-cold PBS, and then 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 μg/ml) followed by Alexa 488 anti-mouse antibodies (1:1000). For copper-treated samples, 100 μM CuCl$_2$ was added to the medium for 2 h prior to fixing.

Detection of hCtr1 Protein Levels at the Plasma Membrane and Western Blots—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 as described previously with minor modifications (6). HEK/hCtr1-myc cells were cultured for 24 h in poly(L-lysine)-coated 6-well trays, washed twice with PBS on ice, and blocked for 10 min with ice-cold PBS containing 5% skim milk. The cells were then incubated with the anti-myc antibody (1:500) for 20 min at 4 °C. After washing the cells five times with 2 ml of ice-cold PBS to remove unbound antibodies, the cells (with retained antibodies) were lysed by sonication in lysis buffer (62 mM Tris-Cl (pH 6.8), 2% SDS, 100 mM dithiothreitol, and protease inhibitor mix (Roche Applied Science)) and then fractionated by 4–20% SDS-PAGE. After transferring proteins to nitrocellulose membranes, the anti-myc antibodies were detected using anti-mouse antibodies conjugated to horseradish peroxidase (HRP) (1:5000) using a chemiluminescence detection kit (Roche Applied Science). Tubulin protein levels were detected on parallel immunoblots using antitubulin antibodies (1:40,000; Sigma). The detection of total myc-tagged wild type and mutant hCtr1 proteins was performed as described previously (6).

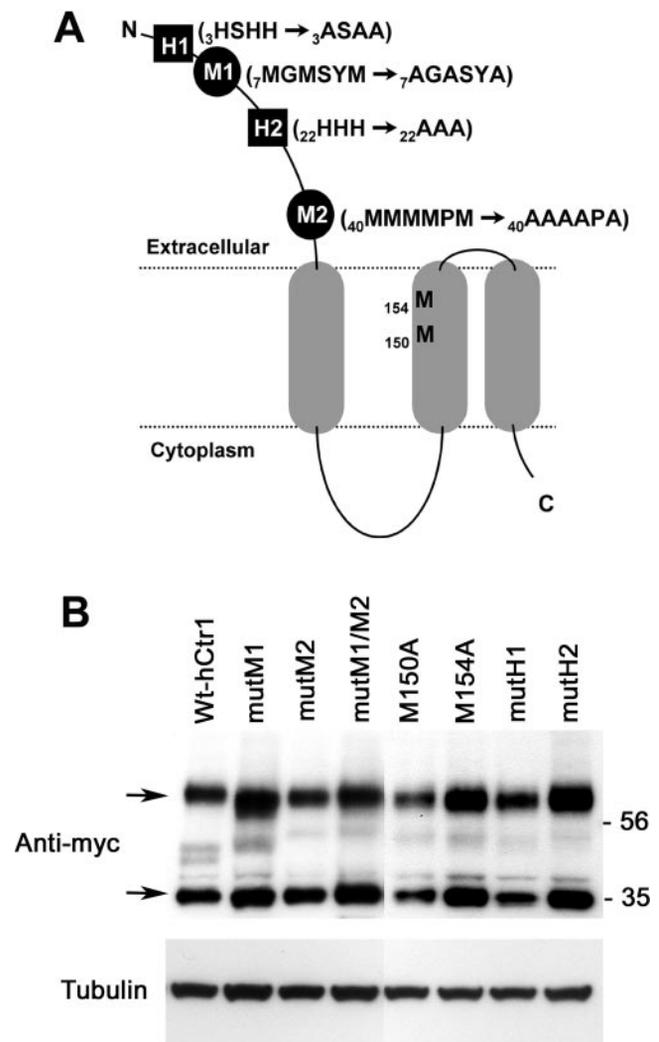*Assay of hCtr1 Endocytosis*—The endocytosis of hCtr1-myc was de-

FIG. 1. **Characterization of HEK293 cell lines expressing hCtr1 mutant proteins.** *A*, schematic diagram of the hCtr1 protein and mutations investigated in this study. Alanine substitutions introduced into the methionine-rich Mets motifs, M1 and M2 (*black circles*), and the histidine-rich sequences, H1 and H2 (*black squares*), are indicated. The Met^{150} and Met^{154} residues of the conserved M*XXX*M motif were replaced by alanine. *B*, Western blot analysis of HEK293 cells stably expressing hCtr1 mutant proteins shown in *A*. Total cell lysates were immunoblotted with anti-myc antibodies to detect hCtr1 protein or with antitubulin antibodies as a control for protein loading. The 35-kDa hCtr1 monomer and 70-kDa multimer are indicated (*arrows*). *Wt*, wild type; *mut*, mutant.

termined by measuring the uptake of anti-myc antibodies added to the cultured medium of stably transfected HEK293 cells as previously described (6). Cells were pregrown in 6-well trays for 24 h in basal medium and then incubated for 5 min at 37 °C in basal or copper-supplemented medium containing 10 μg/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 (100 mM glycine, 20 mM magnesium acetate, 50 mM potassium chloride, pH 2.2). Cells were harvested by scraping into ice-cold PBS and pelleted by centrifugation at 1000 × *g*. The cell pellets were solubilized in SDS buffer (see above), and 20 μg of lysates containing internalized anti-myc antibodies were separated using 4–20% SDS-PAGE, transferred to nitrocellulose membranes, and detected by chemiluminescence using anti-mouse HRP antibodies.

RESULTS

The human Ctr1 protein contains two Mets motifs, M1 (^{7}MGMSYM^{12}) and M2 (^{40}MMMMPM^{45}), and two histidine-rich regions, H1 (^{3}HSHH^6) and H2 (^{22}HHH^{24}), in the extracellular amino-terminal region (Fig. 1*A*). Copper uptake experiments using transfected HEK293 cells and complementation
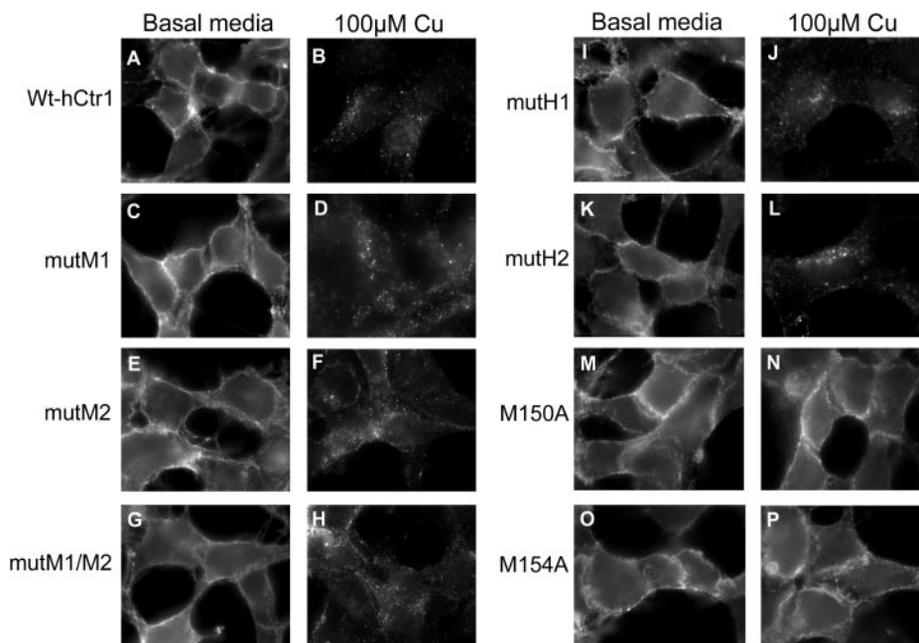
FIG. 2. **Copper-dependent relocalization of the hCtr1 mutant proteins.** Shown is immunofluorescence microscopy analysis of HEK293 cells expressing each hCtr1 mutant protein; the cells were exposed to either basal medium (*A*, *C*, *E*, *G*, *I*, *K*, *M*, and *O*) or medium containing 100 μM copper (*B*, *D*, *F*, *H*, *J*, *L*, *N*, and *P*) for 2 h. The cells were fixed, permeabilized, and blocked prior to detection of wild type (*Wt*) and mutant (*mut*) hCtr1 proteins using anti-myc antibodies followed by anti-mouse Alexa 488 secondary antibodies. Note the severe reduction of copper-induced relocalization of M150A and M154A mutant proteins from the plasma membrane.

studies of the yeast $\Delta ctr1\Delta ctr3\Delta$ mutant suggest that the M2 motif, but not the M1, H1, or H2 motifs, is required for high affinity copper uptake activity under low copper conditions (16). The same study found that both methionines of the conserved ^{150}MXXXM^{154} motif are absolutely critical for copper uptake via hCtr1 but not for localization to the plasma membrane. The aim of the current study was to determine the importance of these motifs for the copper-responsive endocytosis and degradation of hCtr1 observed previously (6). All histidine residues in the H1 and H2 motifs and methionine residues in the M1 and M2 motifs were substituted for alanine, and HEK293 cells were stably transfected with myc-tagged hCtr1 constructs bearing these mutations. After selecting for G418 resistance, surviving populations of cells were screened for expression of the mutant hCtr1 proteins using immunoblots. This enabled the identification of cell lines with similar levels of expression of the monomeric (35-kDa) and multimeric (70-kDa) forms of hCtr1 mutant proteins (Fig. 1*B*).

Immunofluorescence microscopy was used to investigate the steady-state distribution of each hCtr1 mutant protein in cells cultured in basal medium and the ability to relocalize in response to elevated copper. The Wt-hCtr1myc protein was located at the plasma membrane in cells cultured in basal medium (Fig. 2*A*), consistent with previous studies (6). Exposure of the cells to 100 μM copper for 2 h resulted in the relocalization of Wt-hCtr1myc to cytoplasmic vesicles and a clear reduction in fluorescent staining of the cell periphery and overall signal intensity (Fig. 2*B*). This result was consistent with the copper-stimulated endocytosis and degradation of Wt-hCtr1myc protein reported previously (6). Immunofluorescence analysis of the hCtr1 mutant proteins indicated that for each mutation there was strong labeling of the periphery of cells cultured in basal medium conditions, suggesting that none of the mutations grossly interfered with hCtr1 trafficking to the plasma membrane (Fig. 2, *C*, *E*, *G*, *I*, *K*, *M*, and *O*). An incubation for 2 h with 100 μM copper stimulated the relocalization of M1, M2, M1/M2, H1, and H2 mutants to cytoplasmic vesicles (Fig. 2, *D*, *F*, *H*, *J*, and *L*), suggesting that none of these mutations abolished copper-responsive endocytosis of hCtr1 under these high copper conditions (see below for low copper conditions). This result was in sharp contrast to the M150A and M154A mutations, which completely abolished relocalization of hCtr1 following copper treatment as indicated by the strong

fluorescent staining remaining at the periphery of cells (Fig. 2, *N* and *P*). Longer exposure times and higher concentrations of copper failed to alter the location of the M150A and M154A mutant proteins (data not shown). Identical results were obtained when M150 or M154 was replaced with the similarly sized non-polar residue leucine (data not shown), suggesting that the defective trafficking response was specific to the loss of methionine. These findings suggest that the copper-induced relocalization of hCtr1 is dependent on both methionines within the conserved ^{150}MXXXM^{154} sequence.

To examine further the effect of the above mutations on hCtr1 relocalization, we measured levels of each hCtr1 mutant protein remaining at the plasma membrane following copper treatment. The location of the amino-terminal myc tag is extracellular when hCtr1-myc is at the plasma membrane (6, 11, 18). In previous studies we have measured copper-dependent changes in the pool of hCtr1-myc protein at the plasma membrane by probing non-permeabilized cells with anti-myc antibodies followed by Western blotting to determine the levels of these antibodies bound to the cell surface (6). Treatment of cells for 2 h with 100 μM copper resulted in a marked reduction in levels of Wt-hCtr1myc at the plasma membrane (Fig. 3, *lanes 3* and *4*). In control experiments, there was no binding of anti-myc antibodies to the surface of HEK293 cells transfected with the empty vector (Fig. 3, *lanes 1* and *2*). Copper treatment also resulted in a marked reduction in the plasma membrane levels of H1, H2, M1, M2, and M1/M2 mutant proteins (Fig. 3). However, this surface reduction of H2, M1, and M2 mutants occurred to a lesser extent than that of the wild type protein (see "Discussion"). Significantly, the surface levels of the M150A and M154A mutant proteins were not altered by elevated copper concentrations (Fig. 3). This result confirmed our earlier immunofluorescence data for M150A and M154A mutants, which indicated that elevated copper did not alter the steady-state distribution of these proteins (Fig. 2).

Because elevated copper also stimulates the degradation of wild type hCtr1 (6), we tested whether the total abundance of hCtr1 following exposure to elevated copper levels was affected by the mutations depicted in Fig. 1. Western blots were used to detect levels of the hCtr1 mutant proteins in total cell lysates following a 3-h exposure to 100 μM copper in the presence of cycloheximide to prevent new protein expression (Fig. 4). The levels of both 35- and 70-kDa forms of the Wt-hCtr1myc protein
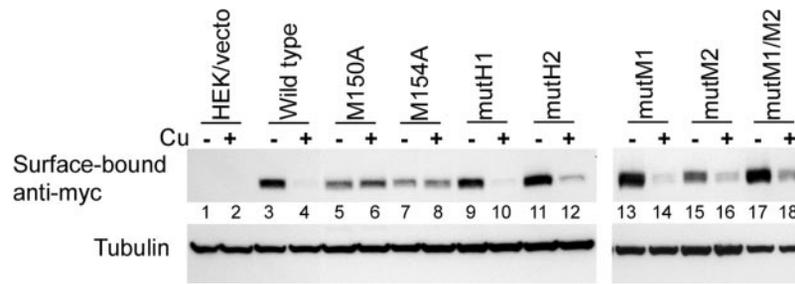
Fig. 3. **Analysis of plasma membrane levels of hCtr1 mutant proteins following copper treatment.** Immunoblots were used to detect anti-myc antibodies bound to the surface of HEK293 cells expressing each hCtr1 mutant protein after exposure to either basal medium (−) or medium containing 100 μM copper (+) for 2 h. Intact cells were cooled on ice and probed with anti-myc antibodies to bind the surface pool of hCtr1. Total lysates were prepared, and anti-myc antibodies were detected using anti-mouse HRP secondary antibodies. Tubulin protein was detected in each sample to indicate equal protein loading. *mut*, mutant.
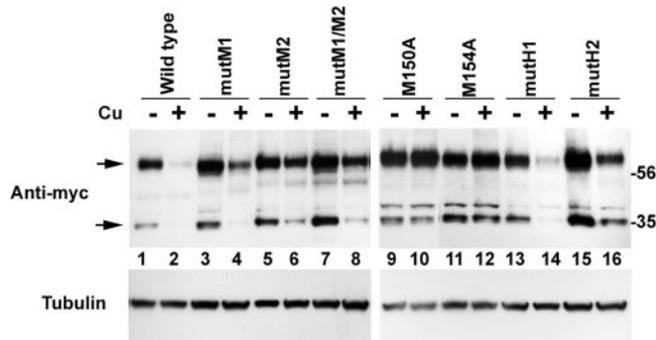

Fig. 4. **Copper-dependent degradation of the hCtr1 mutant proteins.** HEK293 cells expressing each hCtr1 mutant protein were exposed to either basal medium (−) or medium containing 100 μM copper (+) for 3 h prior to immunoblot analysis to detect total hCtr1 protein levels using anti-myc antibodies. Tubulin protein levels demonstrate equal protein loading of samples. *mut*, mutant.

were markedly reduced in response to this copper treatment, and a similar degree of degradation was observed for the H1 mutant protein (Fig. 4, *lanes 13* and *14*). Interestingly, the degradation of the M1, M2, M1/M2, and H2 mutants was clearly apparent in copper-treated cells; however, in each case it was not as extensive as for the Wt-hCtr1myc protein (Fig. 4; see "Discussion"). In contrast, however, the M150A and M154A mutations completely abolished hCtr1 degradation in response to copper treatment (Fig. 4, *lanes 9–12*). Together with the immunofluorescence analysis and assays of surface hCtr1 protein levels, these findings suggest that the Met[150] and Met[154] residues are critical for the copper-stimulated relocalization and degradation of hCtr1.

A more rigorous analysis of copper-stimulated endocytosis was undertaken for each of the hCtr1 mutants by assessing the internalization of anti-myc antibodies added to the culture medium of cells. We have used this method previously to define the kinetics, sensitivity, and metal specificity of copper-stimulated endocytosis of hCtr1-myc (6). A low rate of endocytosis of Wt-hCtr1myc occurred in basal medium, as evident by the weakly detected internalized anti-myc antibodies (Fig. 5, *lane 3*). However, endocytosis of Wt-hCtr1myc was stimulated in the presence of elevated copper as indicated by the increased antibody accumulation (Fig. 5, *lane 4*). The internalization of anti-myc antibodies was dependent on the expression of the hCtr1-myc protein because it did not occur in HEK293 cells expressing the empty pcDNA3.1 vector (Fig. 5, *lanes 1* and *2*). For each of the mutants, anti-myc antibodies were internalized under basal copper conditions, suggesting that none of the mutations abolished the endocytosis of hCtr1-myc. It was notable that the uptake of anti-myc antibodies from basal medium by the M2 mutant protein was lower than for the wild type protein (Fig. 5, *lane 7*). This may be due in part to its lower
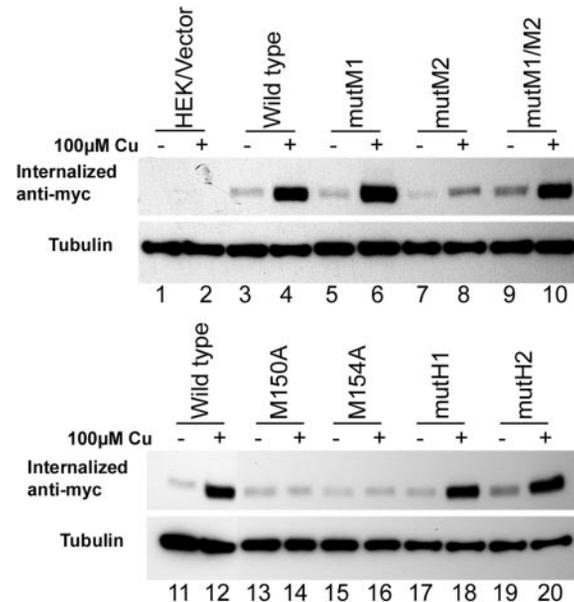

Fig. 5. **Analysis of copper-dependent endocytosis of the hCtr1 mutant proteins.** HEK293 cells stably expressing wild type or mutant (*mut*) hCtr1 proteins were allowed to internalize anti-myc antibodies added to basal medium (−) or medium containing 100 μM copper (+) for 5 min. Surface antibodies were then removed by washing cells in cold acidic buffer, and intracellular antibodies were then detected by immunoblotting with anti-mouse antibodies conjugated to HRP. Note the inhibition of copper-stimulated endocytosis of M150A and M154A mutant proteins.

abundance at the plasma membrane (Fig. 3, *lane 15*), possibly due to a small reduction in its secretion to the plasma membrane. Elevated copper levels stimulated a marked increase in the rate of endocytosis for each hCtr1 mutant protein compared with basal medium, except for the M150A and M154A mutants, which showed no changes in endocytosis. Increasing the copper levels above 100 μM failed to stimulate endocytosis of the M150A and M154A mutants above that observed in basal medium (data not shown). These findings, together with earlier studies, suggested that the Met[150] and Met[154] residues are critical not only for copper uptake but also for the copper-responsive endocytosis and degradation of hCtr1.

We have demonstrated previously that the addition of low micromolar levels of copper to basal medium is sufficient to stimulate the endocytosis of the hCtr1-myc protein (6). Given that the M1, M2, H1, and H2 sequences may provide high affinity copper-binding sites, we investigated whether these motifs were essential for increasing the rate of hCtr1 endocytosis in response to a range of physiologically relevant copper concentrations closer to the K_m value for hCtr1-mediated copper uptake (~2 μM copper). As shown in Fig. 6, the endocytosis
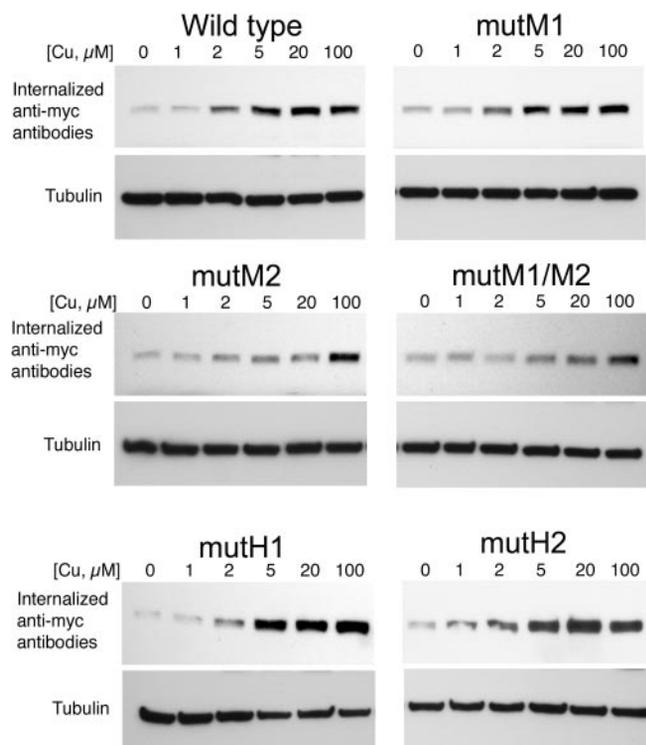
FIG. 6. **The sensitivity of amino-terminal hCtr1 mutants to copper-induced endocytosis.** HEK293 cells stably expressing wild type or mutant (*mut*) hCtr1 proteins were allowed to internalize anti-myc antibodies added to basal medium (*0*) or medium containing a range of copper concentrations for 5 min. Surface antibodies were then removed by washing cells in cold acidic buffer, and intracellular antibodies were then detected by immunoblotting with anti-mouse antibodies conjugated to HRP. Note the higher copper concentrations required to stimulate endocytosis of the M2 and M1/M2 mutant proteins.

of the Wt-hCtr1myc protein was stimulated by 2 μM copper and saturated between 5 and 20 μM copper. A similar dose response was observed for the endocytosis of M1, H1, and H2 mutants (Fig. 6). However, the endocytic response of the M2 mutant protein required copper concentrations greater than 20 μM, suggesting that the sensitivity of this endocytic response was markedly reduced by this mutation. Moreover, the sensitivity of the M1/M2 mutant to low copper levels was also reduced and showed a similar profile to that of the M2 mutant. These findings suggest that the endocytic response of hCtr1 to low micromolar copper levels is dependent on the M2 motif and that the M1 and M2 motifs do not work together in stimulating hCtr1 endocytosis under these copper conditions.

DISCUSSION

The regulated trafficking of the copper transporters ATP7A, ATP7B, and hCtr1 is one of the most acutely controlled aspects of mammalian copper homeostasis (6). The aim of this study was to test whether the amino-terminal H1, H2, M1, or M2 motifs or the putative intramembraneous ^{150}MXXXM^{154} motif of hCtr1 is required for copper-responsive endocytosis. Using a combinatorial approach, our study has demonstrated that both methionine residues of the ^{150}MXXXM^{154} motif are essential for both copper-stimulated endocytosis and degradation of the hCtr1 protein. Significantly, previous studies have shown that this MXXXM motif is also critical for copper uptake via hCtr1, as well as the yeast orthologue yCtr1 (16). Because the sulfur ligand of methionine is a soft Lewis base, the MXXXM motif of the Ctr1 family of proteins has been proposed to constitute a high affinity binding site for copper(I), a soft Lewis acid, during its translocation across the membrane channel (16). Our finding that the MXXXM motif is required for copper-stimulated

endocytosis of hCtr1 suggests that copper binding to this motif during copper transport may also signal endocytosis of the hCtr1 protein (discussed further below).

Immunocytochemical studies indicated that high copper levels (100 μM copper) shifted the steady-state distribution of the M1, M2, M1/M2, H1, and H2 mutant proteins to a vesicular distribution, suggesting that none of these mutations completely abolished relocalization of the hCtr1 protein from the plasma membrane. Consistent with this result was the finding that the levels of the M1, M2, M1/M2, H1, and H2 mutants at the plasma membrane were reduced by this same elevated copper treatment. However, it was notable that the extent of this reduction in surface levels was lower for the H2, M1, M2, and M1/M2 mutants compared with the wild type protein. Similarly, the analysis of total hCtr1 protein levels following copper treatments suggested that these same mutations reduced the extent of hCtr1 degradation relative to the wild type protein and probably contributed to the higher surface levels under these elevated copper conditions. It is currently unclear why these differences in degradation exist for H2, M1, M2, and M1/M2 mutants; however, it is possible that mutation of the M1, M2, and H2 motifs may affect to varying extents hCtr1 endocytosis, intracellular trafficking to degradatory compartments, and/or the degradation process itself.

One of the most significant results of this study was obtained when we examined the endocytic response of the amino-terminal mutants to low copper concentrations. Although the endocytosis of each amino-terminal mutant protein was responsive to high copper levels (100 μM), the M2 mutation was unique in preventing the endocytic response to low copper concentrations. These findings suggested that the M2 motif is not critical for copper-stimulated endocytosis *per se* but is required for this process in response to low copper levels within a physiologically relevant concentration range. It is notable that other studies have shown a similar requirement for the M2 motif for copper transport activity under low copper conditions (16). The M2 mutant protein is able to partially complement the copper-deficient phenotype of the yeast $\Delta ctr1\Delta ctr3\Delta$ mutant, and unlike the wild type hCtr1 protein it is unable to complement under copper-depleted conditions (16). Hence, the M2 motif is required for both high affinity copper transport activity and copper-stimulated endocytosis when copper levels are low. Together with the ^{150}MXXXM^{154} mutation described above, the phenotype of the M2 mutation provides additional evidence suggesting that both copper transport and trafficking functions of hCtr1 may be coupled.

How might the copper-responsive endocytosis and transport functions of hCtr1 be mechanistically coupled? One possibility is that certain hCtr1 conformations associated with its copper transport activity may stimulate endocytosis by triggering interactions between an endocytic motif and sorting machinery on the cytoplasmic face of the plasma membrane. Hence, the ^{150}MXXXM^{154} motif mutations may have blocked copper-responsive endocytosis because these mutations abolish transport activity. Because the M2 mutation prevents high affinity copper transport (16), this may explain why this mutation also inhibited the endocytic response to low copper concentrations.

The transport-dependent model for hCtr1 endocytosis proposed above has remarkable parallels to that recently proposed to explain copper-regulated trafficking of the ATP7A and ATP7B copper ATPases (19). These proteins undergo copper-stimulated trafficking from the trans-Golgi network (2, 4), and like hCtr1, this trafficking response is inhibited by mutations that block copper transport activity (19–21). In the case of ATP7A, the formation of a phosphorylated intermediate as part of the catalytic cycle of the ATPase is required for copper-

stimulated trafficking (19). Support for this model comes from the observation that mutations blocking the formation of this intermediate also block trafficking and that mutations resulting in the accumulation of this intermediate stimulate trafficking (19). The ATP7A and ATP7B proteins possess a CPC motif within the sixth transmembrane domain, which, in the case of ATP7A, is essential for both transport activity and copper-responsive trafficking (20). This CPC motif, which exists as CPC, CPH, or CPS in other heavy metal ATPases, is thought to coordinate the metal as it is translocated through the membrane channel. Hence, the requirement for the CPC motif for both transport and trafficking function is reminiscent of our findings for the ^{150}MXXXM^{154} motif of hCtr1, which is also predicted to reside within a transmembrane region. Other functional similarities between hCtr1 and the copper ATPases are apparent when considering the importance of the amino-terminal regions of these proteins. The ATP7A and ATP7B proteins possess six copper-binding CXXC motifs within their amino-terminal domains that are critical for copper transport activity only under low copper levels (22). In the case of ATP7A, the two motifs closest to the first transmembrane domain are also required for copper-responsive trafficking (23). In these respects the CXXC motif of the copper ATPases bears a striking resemblance to the M2 motif of hCtr1. The significance of these similarities between the copper ATPases and hCtr1 is unclear; however, detailed analysis of the transport mechanisms of both types of copper transporter will likely shed light on how these proteins mediate the trafficking responses to elevated copper concentrations. In summary, we have identified the first critical determinants of copper-responsive endocytosis of the hCtr1 copper transporter. Additional biochemical, structural, and cell biological studies will be required to elucidate the precise steps involved in mediating both copper uptake and the endocytic response to elevated copper as well as whether certain copper-bound conformations exert regulatory control over both these processes.

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