Copper-regulated Trafficking of the Menkes Disease Copper ATPase Is Associated with Formation of a Phosphorylated Catalytic Intermediate*

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The Menkes protein (MNK; ATP7A) is a copper-transporting P-type ATPase that is defective in the copper deficiency disorder, Menkes disease. MNK is localized in the trans-Golgi network and transports copper to enzymes synthesized within secretory compartments. However, in cells exposed to excessive copper, MNK traffics to the plasma membrane where it functions in copper efflux. A conserved feature of all P-type ATPases is the formation of an acyl-phosphate intermediate, which occurs as part of the catalytic cycle during cation transport. In this study we investigated the effect of mutations within conserved catalytic regions of MNK on intracellular localization and trafficking from the trans-Golgi network (TGN). Our findings suggest that mutations that block formation of the phosphorylated catalytic intermediate also prevent copper-induced relocalization of MNK from the TGN. Furthermore, mutations in the phosphatase domain, which resulted in hyperphosphorylation of MNK, caused constitutive trafficking from the TGN to the plasma membrane. A similar effect on trafficking was observed with a phosphatase mutation in the closely related copper ATPase, ATP7B, affected in Wilson disease. These findings suggest that the copper-induced trafficking of the Menkes and Wilson disease copper ATPases is associated with the phosphorylated intermediate that is formed during the catalysis of these pumps. Our findings describe a novel mechanism for regulating the subcellular location of a transport protein involving the recognition of intermediate conformations during catalysis.

Intracellular copper levels must be tightly controlled, as a deficiency or excess of copper is deleterious to cell survival. The requirement for intact cellular copper transport systems in humans is highlighted by two genetic disorders of copper metabolism, X-linked Menkes disease and autosomal recessive Wilson disease (1). These genetic disorders are caused by mutations in distinct but closely related copper-transporting P-type ATPases, known as the Menkes protein (ATP7A or MNK) and the Wilson protein (ATP7B or WND). P-type ATPases are a family of ATP-driven cation transporters present in all phyla, whose various substrates include H⁺, Ca²⁺, Mg²⁺, Na⁺, K⁺, Cd²⁺, Pb²⁺, Zn²⁺, Ag⁺, and Cu⁺ (2). These proteins hydrolyze ATP and use the γ-phosphate to form an acyl-phosphorylated intermediate, which is subsequently dephosphorylated by an intrinsic phosphatase activity. This cycle of phosphorylation and dephosphorylation provides the energy for cation translocation across lipid bilayers (3). The MNK and WND proteins contain the hallmark sequences for ATP binding, phosphorylation, and phosphatase reactions; however, additional sequences have further classified MNK and WND proteins as heavy metal-transporting CPx-ATPases (4). These additional sequences include Cys-Pro-Cys (CPC) in the sixth membrane-spanning domain, a His-Pro (HP) dipeptide in the third cytoplasmic loop, and one or more amino-terminal metal binding motifs containing the core sequence, Cys-X-X-Cys (see Fig. 1A) (4).

Studies in cultured cells have shown that MNK and WND proteins are copper exporters (5, 6). The proteins differ in their tissue distribution of expression; WND is predominantly expressed in the liver (7), whereas MNK is present in most non-hepatic tissues (8). In Menkes patients, a defect in copper export from intestinal cells and aberrant distribution of copper around the body causes a systemic copper deficiency, with symptoms including severe neurological and connective tissue defects, impaired temperature control, and hypopigmentation (1, 9). The WND protein is required for the export of excess hepatic copper into the bile, and Wilson patients suffer a toxic build-up of hepatic copper and cirrhosis of the liver (1).

Immunocytochemical studies have shown that both the MNK and WND ATPases reside in the final compartment of the Golgi apparatus, the trans-Golgi network (TGN) (7, 10–12). At this location, MNK and WND transport copper to the lumen of secretory compartments to provide copper to various copper-dependent enzymes (13, 14). We have shown previously that in the presence of elevated copper the MNK protein traffics to the plasma membrane, where it maintains copper homeostasis via copper efflux (11, 15). In hepatocytes, copper induces the relocalization of WND to cytoplasmic vesicles associated with the canalicular membrane of bile ducts (12). In non-hepatic cell types, WND relocates to an undefined population of cytoplasmic vesicles that may represent a novel copper storage/detoxification compartment (7, 16). The copper-induced trafficking of MNK and WND serves to shift the function of these copper ATPases from their role in the biosynthesis of...
cuproenzymes in the TGN to copper export and the prevention of copper toxicity in post-TGN compartments. More recently, the regulated trafficking of the zinc importer, Zrt1 (17), and the copper importer, Ctrl (18), in response to changing levels of their respective metal ligands, has been identified in the yeast Saccharomyces cerevisiae. Hence, the regulated trafficking of metal transporters is likely to be a general means by which metal homeostasis is regulated in all eukaryotic cells. So far there have been no studies on any transporter that address the mechanisms by which metal ions are sensed and how this information is coordinated with a trafficking response.

Previous studies have identified several disease-causing mutations in MNK and WND that prevent the copper-induced trafficking from the TGN (16, 19–23). These mutations do not cluster within a distinct region, suggesting that the alterations are unlikely to directly affect copper-induced trafficking per se. Rather, we hypothesize that the trafficking of MNK and WND is dependent on copper transport activity and that these mutations indirectly prevent trafficking by affecting catalysis. The aim of this study was to explore this potential association between catalysis and trafficking by introducing disease-causing mutations into the MNK protein that occur in highly conserved sequences and were either known or predicted to abolish catalytic copper transport function. Mutations affecting the conserved CPC and HP sequences, and the phosphorylated aspartic acid inhibited copper-induced trafficking from the TGN. However, mutations within the phosphate domain resulted in the constitutive relocalization of MNK to the plasma membrane without additional copper. These constitutive trafficking mutations were also associated with hyperphosphorylation of MNK. Our findings suggest that it is the formation of the phosphorylated intermediate during MNK catalysis that serves to trigger the exocytic trafficking of the transporter from the TGN. These findings identify a novel mechanism underlying the trafficking of a transport protein, whereby catalytic activity regulates the distribution between subcellular compartments.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Chinese hamster ovary (CHO) cell lines were maintained in minimum Eagle’s medium containing 10% fetal bovine serum and supplemented with 2 mM l-proline and penicillin/streptomycin in a 5% CO₂, 37 °C incubator. Affinity-purified MNK antibodies were raised in sheep against the amino-terminal copper binding domain of MNK and WND (Stratagene). Mutant clones were verified by DNA sequencing.

Immunofluorescence and Immunoblotting—Immunofluorescence microscopy was performed as described previously (24). In some experiments, cells were cultured for 4 h in media containing 200 μM CuCl₂ (+Cu condition). The basal copper condition was growth media without additional copper (~0.5 μM copper). Alexa 594-conjugated anti-sheep antibodies and Alexa 488-conjugated anti-rabbit antibodies (Molecular Probes, Eugene, OR) were diluted 1:1000 and used to detect primary antibodies. Immunoblotting was performed as described previously (15).

Phosphorylation Assays—Phosphorylation assays were performed using purified vesicles from yeast Δcpc2 transformants, as we have described recently (27). MNK phosphorylation assays were conducted using 20 μg of vesicle protein on ice in 20 mM MOPS buffer (pH 6.8), 150 mM NaCl, 5 mM MgCl₂, 50 μM dithiothreitol, and 5 μM CuCl₂. Phosphorylation reactions proceeded for 20 s after pulsing with 1 μM [γ-³²P]ATP (10 Ci/nmol, GeneWorks). The MNK protein was immunoprecipitated, subjected to SDS-PAGE, and detected by autoradiography as we have described previously (27). In some experiments, the copper chelator bathocuproine disulfonate (1 mM), or the phosphorylation inhibitor sodium vanadate (200 μM), were added to reactions (27).

RESULTS

The Effect of Disease-causing Mutations on the Subcellular Location of the MNK Protein—We began this study by investigating whether copper-induced trafficking of the MNK protein is dependent on a functional copper transporter. Our initial strategy was to generate a series of missense mutations in highly conserved regions of MNK predicted to be functionally important and test their effects on subcellular location and copper-induced trafficking. The first mutation tested was C396R in MNK, which is conserved in all CPx-ATPases and located between 39 and 43 amino acids downstream of the histidine-proline dipeptide, which is conserved in all CPx-ATPases. Mutations in the TGE cluster within a distinct region, suggesting that the alterations indirectly prevent trafficking by affecting catalysis. The activity regulates the distribution between subcellular compartments.
In basal copper conditions the mutant proteins, MNK-C1000R and MNK-H1086Q, were detected in the perinuclear region of cells (Fig. 2, C and E). As we have reported previously for WtMNK (11), the treatment of cells with brefeldin A caused contraction of both MNK-C1000R and MNK-H1086Q to a tight juxtanuclear spot (data not shown), which is a specific effect of brefeldin A on proteins of the TGN. These data suggested that neither C1000R nor H1086Q mutations interfered with the TGN localization of MNK. However, in elevated copper both the MNK-C1000R and MNK-H1086Q proteins remained in the TGN and did not traffic to the plasma membrane (Fig. 2, D and F). Increases in the copper concentration or exposure time did not change this perinuclear location of MNK-C1000R and MNK-H1086Q proteins (data not shown). These observations were consistent with the hypothesis that copper-induced trafficking of MNK is dependent on a functional copper transporter.

A surprising result was obtained when we investigated the distribution of the MNK-L873R protein. Rather than being TGN-localized under basal copper conditions, the L873R mutant was associated predominantly with the plasma membrane (Fig. 2G). This distribution differed from the perinuclear location of WtMNK, C1000R, and H1086Q proteins in basal medium and resembled that of the WtMNK protein under elevated copper conditions (Fig. 2B). Elevated copper treatment did not change the apparent distribution of the MNK-L873R protein (Fig. 2H). The leucine 873 residue lies two amino acids away from the TGE phosphatase domain (Fig. 1A). Mutation of the TGE motif in the sarcoplasmic endoplasmic reticulum calcium pump causes hyperphosphorylation by inhibiting turnover of the E1-P conformation of the enzyme (34). This information highlighted a possible connection between MNK phosphorylation and its exocytic trafficking. Therefore, we speculated that the plasma membrane location of the L873R mutant protein may be because of the accumulation of the MNK-phosphorylated catalytic intermediate. To this test hypothesis more directly, we investigated the effect of mutations within the phosphorylation site and the phosphatase domain on MNK phosphorylation and trafficking.

**The Subcellular Localization of MNK with Mutations in Phosphorylation and Phosphatase Domains**—The aspartic acid residue within the signature sequence, DKTGT, is the site of acyl-phosphate formation in all P-type ATPases (36). Studies involving the expression of MNK in the yeast *S. cerevisiae* have shown that the aspartic acid in the DKTGT motif is essential for copper transport (27). This yeast copper transport assay involves expression of MNK in the Δccc2 mutant, which lacks the MNK homologue, Ccc2. In the absence of Ccc2, the Δccc2 mutant is unable to grow on copper- and iron-limited medium, because copper transport by Ccc2 to the secreted cuproenzyme, Fet3, is essential for high affinity iron uptake. The expression
of MNK complements the poor growth of the Δccc2 mutant on media limited in copper and iron (27, 32). Vesicle preparations from the MNK-expressing Δccc2 mutant have shown that MNK forms an acyl-phosphate intermediate and that this is dependent on the Asp1044 residue in the DKTGT motif (27). We confirmed these previous findings in our study by showing complementation of the Δccc2 growth defect with expression of the wild-type MNK protein (Fig. 3C) and the formation of a phosphorylated intermediate (Fig. 3A). As expected, the D1044E mutation inhibited the formation of the phosphoenzyme (Fig. 3A) and failed to complement the defective growth phenotype of the Δccc2 mutant (Fig. 3C). The phosphorylation of the Δ75TGE-AAA protein was elevated relative to the WtMNK protein (Fig. 3A), consistent with the predicted role of the TGE motif in dephosphorylation during catalysis (34, 35). Significantly, the L873R mutant, which was constitutively located on vesicles and at the plasma membrane (Fig. 2), also caused hyperphosphorylation of MNK (Fig. 3A). The hyperphosphorylation of Δ75TGE-AAA and L873R mutants was consistent with the predicted importance of the TGE motif in dephosphorylation of P-type ATPases. The phosphorylation of Δ75TGE-AAA and L873R mutants was inhibited by the copper chelator, bathocuproine disulfonic acid, and the classical inhibitor of P-type ATPase phosphorylation, vanadate. These observations suggest that both Δ75TGE-AAA and L873R mutants failed to complement the growth defect of the Δccc2 mutant (Fig. 3C), and this was consistent with these mutations blocking the catalytic copper transport cycle.

We then explored the relationship between phosphorylation and copper-induced trafficking of MNK after stably expressing the D1044E and Δ75TGE-AAA mutant proteins in CHO cells. In basal copper conditions, the MNK-D1044E protein was located in the perinuclear region (Fig. 4C) and the formation of a phosphorylated intermediate (Fig. 3A) and failed to complement the defective growth phenotype of the Δccc2 mutant (Fig. 3C). The phosphorylation of the Δ75TGE-AAA protein was elevated relative to the WtMNK protein (Fig. 3A), consistent with the predicted role of the TGE motif in dephosphorylation during catalysis (34, 35). Significantly, the L873R mutant, which was constitutively located on vesicles and at the plasma membrane (Fig. 2), also caused hyperphosphorylation of MNK (Fig. 3A). The hyperphosphorylation of Δ75TGE-AAA and L873R mutants was consistent with the predicted importance of the TGE motif in dephosphorylation of P-type ATPase proteins. The phosphorylation of Δ75TGE-AAA and L873R mutants was inhibited by the copper chelator, bathocuproine disulfonic acid, indicating that this phosphorylation was copper-dependent (Fig. 3B). Moreover, vanadate, a specific inhibitor of aspartyl-phosphate formation in P-type ATPases, impaired the phosphorylation of the TGE-AAA and L873R mutants (Fig. 3D). These observations suggest that both Δ75TGE-AAA and L873R mutations caused hyperphosphorylation of MNK and that copper is required as a substrate to initiate this process. Both the Δ75TGE-AAA and L873R mutants failed to complement the growth defect of the Δccc2 mutant (Fig. 3C), and this was consistent with these mutations blocking the catalytic copper transport cycle.

We then explored the relationship between phosphorylation and copper-induced trafficking of MNK after stably expressing the D1044E and Δ75TGE-AAA mutant proteins in CHO cells. In basal copper conditions, the MNK-D1044E protein was located in the perinuclear region (Fig. 4C), as found for the wild-type MNK protein (Fig. 4A). However, in cells exposed to 200 μM copper for 4 h, MNK-D1044E remained perinuclear (Fig. 4D). No difference to this location was observed with increased copper levels or exposure times (up to 400 μM Cu and 72 h; data not shown). These findings suggested that the copper-induced trafficking of MNK requires the formation of the acyl-phosphate catalytic intermediate. Significantly, in basal medium the MNK(Δ75TGE-AAA) protein was constitutively located at the plasma membrane (Fig. 4E), similar to the effect of the L873R mutation shown earlier (Fig. 2G). No difference in distribution was observed with addition of excess copper to the growth medium (Fig. 4F). Hence, both the Δ75TGE-AAA and
L873R mutations caused constitutive relocalization of MNK from the TGN and caused hyperphosphorylation of MNK in vitro. This was in sharp contrast to the D1044E mutation, which inhibited both the phosphorylation and trafficking response of MNK. Taken together, these findings suggest that copper-induced trafficking of MNK from the TGN to the plasma membrane requires a catalytically active enzyme and is associated with formation of the phosphorylated catalytic intermediate.

Our studies showed that severe copper limitation in vitro inhibited phosphorylation of both 875TGE-AAA and L873R mutants (Fig. 3B). To further explore the correlation between catalysis and trafficking in vivo, we tested whether copper limitation would prevent the plasma membrane distribution of the 875TGE-AAA and L873R mutants. Reduced levels of both mutant proteins at the plasma membrane were observed in cells passaged several times in media depleted of copper with chelators; however, cell viability was also compromised (data not shown). It is important to note that in addition to inhibiting MNK catalysis, copper depletion is also expected to reduce the activity of cytochrome c oxidase, a copper-dependent enzyme involved in generating cellular ATP. Moreover, ATP is directly required for the MNK catalytic reaction cycle, as well as being an indirect energy source for protein trafficking. These potential secondary effects made analysis of copper depletion on the location of the phosphatase mutant difficult to interpret.

Therefore, we chose a genetic approach for testing the catalysis-dependent trafficking model. We reasoned that if the hyperphosphorylation of the 875TGE-AAA mutant triggered trafficking to the plasma membrane, then a second mutation of the aspartic acid phosphorylation site to make a double mutant should suppress this relocalization. A double mutant containing both the D1044E and 875TGE-AAA mutations, MNK(D1044E/TGE-AAA), was stably expressed in CHO cells. The location of the MNK(D1044E/TGE-AAA) protein in cells in basal media was exclusively perinuclear (Fig. 4G) and failed to undergo relocalization to the plasma membrane in cells exposed to elevated copper (Fig. 4H). Thus, the D1044E mutation suppressed the 875TGE-AAA mutation, as would be predicted if copper-induced trafficking of MNK requires formation of a phosphorylated catalytic intermediate.

Mutation of the Phosphatase Domain in the Wilson Disease Copper ATPase Leads to a Constitutive Trafficking Response—The Wilson disease copper transporting P-type ATPase (WND or ATP7B) is located within the TGN in basal copper conditions. However, the WND protein traffics to cytoplasmic vesicles rather than the plasma membrane in response to elevated copper (7, 23). To explore whether copper-induced trafficking of the WND ATPase occurs via a similar mechanism to MNK, we tested whether mutation of the 858TGE sequence of WND would trigger a constitutive trafficking response. Immunofluorescence microscopy of transfected CHO cells confirmed previous studies and showed WtWND to be located in the perinuclear region under basal copper conditions (Fig. 5A) and relocalized to cytoplasmic vesicles in response to elevated copper (Fig. 5B). Significantly, the WND(TGE-AAA) protein was present in a population of cytoplasmic vesicles under basal copper conditions (Fig. 5C), which resembled the distribution of WtWND in cells exposed to elevated copper (Fig. 5D). There was no change to the vesicular staining of the WND(TGE-AAA) protein upon exposure of cells to elevated copper (Fig. 5D). Hence, the TGE mutation in WND appeared to mimic the effect of elevated copper on the wild-type protein and was analogous to the plasma membrane localization observed for the TGE mutation in MNK. Collectively, these data suggest that a similar mechanism underlies the copper-induced trafficking of both MNK and WND proteins, which involves the formation of the acyl-phosphate intermediate during catalysis.
DISCUSSION

Disease-causing mutations in MNK and WND have been shown previously to prevent copper-induced trafficking from the TGN. MNK trafficking-defective mutations include A1364V in the seventh transmembrane domain (19), a deletion of 803AL in second cytoplasmic loop (20), and mutations in the amino-terminal copper binding domains (21, 22). WND trafficking mutations include G943S in the fifth transmembrane region (23), mutation of the conserved 883CPC motif within the sixth transmembrane domain (23), and M1356V in the eighth transmembrane region (16). These studies indicated that mutations affecting copper-induced trafficking do not appear to cluster within a single region and thus did not reveal a single distinct motif essential for trafficking. We hypothesized that trafficking is coupled with catalysis of MNK and WND and that mutations that impair copper transport activity also prevent trafficking. To explore this hypothesis further, we introduced three disease-associated mutations into sequences of MNK that are highly conserved in heavy metal ATPases. The C1006R and H1086Q mutations blocked copper-induced trafficking of MNK from the TGN; however, L873R caused constitutive localization of MNK at the plasma membrane under basal and elevated copper conditions. The close proximity of the L873R mutation to the TGE phosphatase domain provided a clue to the possible association of copper-induced trafficking of MNK with formation of phosphorylated catalytic intermediate.

We found that mutation of the phosphorylation site of MNK impaired both the copper-induced trafficking to the plasma membrane and the formation of the phosphorylated intermediate. However, the TGE-AAA and L873R mutations resulted in hyperphosphorylation, and this was associated with a constitutive relocalization from the TGN. These findings suggest that formation of the acyl-phosphate intermediate is required for trafficking of MNK, and by locking the protein in the phosphorylated state, a constitutive trafficking response occurs even in basal copper conditions. Moreover, the finding that the double mutant D1044E/TGE-AAA inhibited copper-regulated trafficking from the TGN demonstrated that the plasma membrane location of the TGE-AAA mutation alone was dependent on catalytic phosphorylation of MNK at Asp1044. This TGE-AAA localization was unlikely to have arisen through protein misfolding and secretion to the plasma membrane and was consistent with the phosphorylation-dependent trafficking model.

The analysis of the phosphatase mutation in the WND ATPase provided further evidence for a link between the formation of a phosphoenzyme intermediate and copper-induced trafficking. The WND phosphatase mutation caused constitutive relocalization from the TGN to a vesicular distribution in basal medium, which appeared similar to that induced by elevated copper for the wild-type WND protein. Thus, the phosphatase domain mutations in both MNK and WND result in relocalization from the TGN. But rather than both proteins trafficking to the plasma membrane, these proteins were targeted to destinations that were similar to those of their respective wild-type proteins under elevated copper conditions and suggested preservation of sorting signals for targeting MNK and WND to the appropriate post-TGN trafficking pathways.

How does this model of phosphorylation-dependent trafficking explain the copper-induced relocalization of MNK and WND from the TGN? The copper-induced trafficking of MNK and WND proteins presumably switches the function of these ATPases from their nutritional roles in metallating secreted cuproenzymes to protective roles in the export of excess copper in post-Golgi compartments and the plasma membrane. Our studies suggest that a key aspect of this switch in function is the catalytic cycle of the ATPase itself. It is unclear at this stage whether exocytic sorting of MNK and WND into vesicles at the TGN is directly coupled with formation of the phosphorylated intermediate or whether this sorting process involves additional downstream conformations or modifications. Moreover, the adaptor proteins that are likely to be involved in recognizing MNK and WND and recruiting these transporters into vesicles have not been identified. However, the identification of mutations in this study that either prevent or constitutively induce this trafficking response provides exciting new tools for identifying these sorting proteins.

It is important to note that the catalysis.dependent trafficking model does not predict that MNK and WND will undergo trafficking from the TGN with every catalytic cycle. Several factors that may determine whether a catalytically active transporter will be recruited into an exocytic vesicle include correct localization within a distinct subcompartment of the TGN for protein sorting or the affinity of the ATPases for components of the protein sorting machinery. Indeed, it is likely that a proportion of MNK and WND protein must be retained in the TGN or earlier secondary compartments to complete catalytic cycling without undergoing exocytosis to load copper onto secreted cuproenzymes (13, 14).

The catalysis-dependent trafficking model suggests that the steady state TGN location of MNK and WND under low copper conditions is a reflection of the small proportion of molecules cycling through phosphorylated catalytic intermediates. The shift in distribution of MNK and WND from the TGN upon exposure to elevated copper can be explained by an increase in the proportion of catalytically active proteins and thus the number of transporters cycling through the phosphorylated conformation. By coupling the trafficking of the copper ATPases with catalytic activity, this would allow an appropriate equilibrium of the ATPases to be established between the TGN and post-TGN compartments following dietary copper intake. Moreover, this would provide autoregulation of the trafficking response. In this scenario, the trafficking of MNK and WND proteins to post-TGN compartments would allow the removal of excess copper. This would then lower copper levels to the point where catalytic turnover is insufficient to maintain a steady-state post-TGN distribution, and the equilibrium is ultimately shifted back to the TGN.

In summary, our findings provide the first molecular insights into the mechanism by which the mammalian copper ATPases traffic from the TGN in response to copper. The regulation of subcellular distribution of a transport protein through a mechanism involving recognition of catalytic intermediates is unlike other known regulated trafficking mechanisms. It remains to be shown whether a similar strategy has been adopted for the metal-regulated trafficking of other proteins such as the zinc importer, Zrt1, and the copper importer, Ctr1, and the prion disease protein, PrP (17, 18, 37).

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