The Role of GMXCXXC Metal Binding Sites in the Copper-induced Redistribution of the Menkes Protein*

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The Menkes protein (MNK or ATP7A) is a transmembrane, copper-transporting CPX-type ATPase, a subgroup of the extensive family of P-type ATPases. A striking feature of the protein is the presence of six metal binding sites (MBSs) in the N-terminal region with the highly conserved consensus sequence GMXCXXC. MNK is normally located in the trans-Golgi network (TGN) but has been shown to relocalize to the plasma membrane when cells are cultured in media containing high concentrations of copper. The experiments described in this report test the hypothesis that the six MBSs are required for this copper-induced trafficking of MNK. Site-directed mutagenesis was used to convert both cysteine residues in the conserved MBS motifs to serines. Mutation of MBS 1, MBS 6, and MBSs 1–3 resulted in a molecule that appeared to relocalize normally with copper, but when MBSs 4–6 or MBSs 1–6 were mutated, MNK remained in the TGN, even when cells were exposed to 300 μM copper. Furthermore, the ability of the MNK variants to relocalize corresponded well with their ability to confer copper resistance. To further define the critical motifs, MBS 5 and MBS 6 were mutated, and these changes abolished the response to copper. The region from amino acid 8 to amino acid 485 was deleted, resulting in mutant MNK that lacked 478 amino acids from the N-terminal region, including the first four MBSs. This truncated molecule responded normally to copper. Moreover, when either one of the remaining MBS 5 and MBS 6 was mutated to GMXSXXS, the resulting proteins were localized to the TGN in low copper and relocalized in response to elevated copper. These experiments demonstrated that the deleted N-terminal region from amino acid 8 to amino acid 485 was not essential for copper-induced trafficking and that one MBS close to the membrane channel of MNK was necessary and sufficient for the copper-induced redistribution.

Copper is an essential element required for enzymes such as cytochrome c oxidase, lysyl oxidase, and dopamine-β-hydrox-ylase that employ its fundamental redox properties in the respiratory chain, connective tissue biosynthesis, and catecholamine production, respectively. Nevertheless, these biochemically useful redox properties can also lead to serious cellular damage through the formation of highly reactive free radicals. Thus, intracellular copper concentrations must be tightly regulated. Various molecules are involved in the maintenance of cellular copper homeostasis, many of which have been recently identified and characterized (for a review, see Ref. 1). Among these are copper efflux proteins whose essential role is demonstrated in the genetically inherited Menkes and Wilson diseases. The genes affected in these diseases were identified by several independent groups (2–6) and designated MNK (ATP7A) andWND (ATP7B). MNK and WND encode highly homologous membrane proteins of 178 and 165 kDa, respectively. They belong to the family of P-type ATPases classically represented by the human Ca2+-ATPases and the ubiquitous Na+/K+-ATPases (7). Together with Cd2+-ATPases, Cu2+/Zn2+-ATPases form a subfamily of CPX-type ATPases (X = Cys, His, or Ser) characterized by a conserved Cys-Pro-X motif in the proposed ion transduction channel and a variable number of GMXCXXC putative heavy metal binding sites (MBS) at the N terminus (8). Bacterial copper transporters contain a single MBS (9), yeast possess two MBSs (10), nematodes have three MBSs (11), and the human MNK and WND have six metal binding sites (8). The same motifs are also present in cadmium transporters and in mercury-binding proteins (12), underscoring the role of these motifs in specifically complexing heavy metal ions.

Elucidating the function of the N-terminal GMXCXXC motifs of both WND and MNK has been the subject of several studies (13–16). These studies suggest a binding stoichiometry of one copper atom per metal binding site for both MNK and WND N termini and a capacity to bind Zn, Ni, and Co (13, 14). Thus far, the biochemical function of the MNK and WND N termini has only been studied in a Δcux2 mutant yeast strain in which human MNK and WND fully restored copper delivery to the high affinity iron uptake system (15, 16). The third MBS was found to be most critical for catalytic activity of MNK and was suggested to be involved in protein-protein interaction (15). A further study demonstrated that the deletion of a region comprising MBS 6 rendered WND inactive (16).

In CHO-K1 cells, human fibroblasts, and HeLa cells, MNK is localized to the trans-Golgi network (TGN) (17–19). Copper-dependent relocalization of MNK from the TGN to the plasma membrane was demonstrated in copper-resistant CHO-K1 cells.

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‡§ The abbreviations used are: MBS, metal binding site; MNK, Menkes protein; TGN, trans-Golgi network; CHO, Chinese hamster ovary; kb, kilobase(s); BME, Eagle’s basal media; wt, wild-type; WND, Wilson protein.
that overexpressed the endogenous hamster MNK homologue (17). Recently, this observation was supported by experiments with stable CHO-K1 cells expressing human MNK from a cDNA construct (20). Copper-induced movement of WND from the TGN to an as yet unidentified intracellular compartment has also been observed (21–23). Despite these data, little is known about the structural domains involved in the exocytic and endocytic trafficking of these copper-transporting proteins. An obvious candidate region that may be involved in the copper-dependent trafficking of MNK is the N-terminal region containing the six copper-binding GMXCXXC motifs.

The objective of this study was to clarify the role of the N-terminal MBSs of MNK in copper-induced redistribution. We report here that not all of the MBSs are essential for the relocation of MNK and for conferring a copper-resistant phenotype. Furthermore, the presence of only one MBS within a region of ~170 amino acids adjacent to the proposed transduction channel is necessary and sufficient for the copper-induced exocytic trafficking of the Menkes protein. In addition, the data indicate that protein-protein interactions that may be required for the translocation of MNK from the TGN to the plasma membrane do not involve amino acids 8–485 of the MNK N terminus.

**EXPERIMENTAL PROCEDURES**

**Antibody Production**—The production of the affinity-purified antibody directed against the first 590 amino acids of the MNK N terminus was described previously (24). Rabbit antibodies directed against the C-terminal peptide of amino acids 807–1006 from amino acid 8 to amino acid 485, thus eliminating the first four MBSs.

**Site-Directed Mutagenesis**—To generate MNK mutated in all six MBSs, a mutant MBS 5 was isolated from this plasmid (pCM145) as a XbaI/SalI fragment and cloned into pCM147 to replace the 1.8-kb MBS-encoding fragment. The truncated MNK construct with a mutated MBS 5 was isolated from this plasmid (pCM145) as a XbaI/SalI fragment and cloned into pCM147 to generate the final construct, pCM146.

**Cell Culture and Transient Transfection Experiments**—Cell culture conditions were as described previously (28). Approximately 3 × 10⁵ CHO-K1 cells were seeded onto coverslips on the day before the experiment. Cells were grown to 80% confluency and transfected with the DNA plasmid constructs at 1 μg of supercoiled plasmid DNA and 3 μl of LipofectAMINE (Life Technologies, Inc.) was used in serum-free BME. Transfection procedures and culturing conditions were as described by the manufacturer. At approximately 18 h after transfection, CuCl₂ was added to a final concentration of 200 μM, and cells were incubated for 3 h at 37 °C. Cells were then fixed in acetone and processed for immunofluorescence analysis as described previously (17).

**Immunofluorescence Microscopy**—Cells were grown to ~50% confluence, and transfected cells were carried out as described for transient transfections in a total volume of 5 ml of serum-free BME. Cells were allowed to recover overnight in BME containing 10% fetal calf serum, detached, and seeded into 24-well plates. Selection in 400 μg/ml G418 (Life Technologies, Inc.) was initiated and continued for 12 days. G418-resistant clones were screened for MNK expression by immunofluorescence analysis, and MNK-positive colonies were clonally purified.
licate and allowed to attach overnight. The cells were incubated in BME and 10% fetal calf serum containing 0–105 μM CuCl₂ for the next 7 days. After this period, the cells were fixed in 90% methanol and 10% formaldehyde and stained with 10% Giemsa’s stain (improved R66 solution; Gurr; Merck), and the number of colonies in each Petri dish was counted.

RESULTS

The role of the six MBSs in MNK trafficking was investigated using site-directed and deletion mutagenesis of the MNK N terminus. Constructs encoding MNK mutated in MBS 1 alone (MNKm1), MBS 6 alone (MNKm6), MBSs 1–3 (MNKm1-3), MBSs 4–6 (MNKm4-6), and MBS 5 and MBS 6 (MNKm5-6) were generated. Amino acids 8–485 at the N terminus of MNK were deleted to create MNKD1-4, and in this construct, mutations were separately introduced within MBS 5 (MNKD1-4m5) and MBS 6 (MNKD1-4m6) to generate constructs with only one functional MBS (Fig. 1).

All of the mutated MNK cDNA constructs were initially transiently transfected into CHO-K1 cells, and the intracellular distribution of the mutated MNK molecules was assessed by indirect immunofluorescence using polyclonal antibodies directed against the MNK N or C terminus. Stable cell lines were established for constructs containing MNKm1-3, MNKm4-6, and MNKm1-6. In basal copper concentrations, fluorescent staining was observed within the perinuclear region of all of the transfected cells (Figs. 2–6; Cu), consistent with location at the TGN and with previous results obtained with CHO-K1 cells (17, 20). Thus, the overexpressed, mutated MNKs were not mislocalized under basal conditions. Furthermore, the mutations did not affect MNK epitope recognition by the polyclonal antibody, suggesting that even multiple point mutations within the N terminus did not induce significant antigenic changes in the molecule.

After incubation in 200 μM copper for 3 h, transiently expressed human MNK that was mutated in either MBS 1 (MNKm1) or MBS 6 (MNKm6) was redistributed and was clearly present within the cytoplasm and at the plasma membrane (Fig. 2). There were no obvious differences in the staining pattern of cells expressing mutated or wt proteins as observed after incubation in different copper concentrations (data not shown). Thus, a single GMXCS mutation within the MNK N terminus did not influence the copper-inducible relocation of MNK.

Proteins transiently expressed in cells differ significantly in their levels of expression. Therefore, the interpretation of results obtained with transiently transfected cells can be complicated by artifacts resulting from overexpression of the target protein. To allow a clearer analysis of the effects of mutations, stable cell lines were generated from CHO-K1 cells that expressed the mutant MNK forms MNKm1-3, MNKm4-6, and MNKm1-6. These cell lines were designated 114, 115, and 116, respectively.

To confirm that the expressed mutant proteins were of the expected sizes and to determine the level of MNK expression, Western blots on whole cell lysates of the stable cell lines were carried out (Fig. 3A). A protein of the expected size of ~178 kDa was detected in each of cell lines 114, 115, and 116, also demonstrating the capacity of the anti-MNK antibody to recognize the mutated proteins. Compared with the endogenous CHO-K1 Menkes protein homologue, significantly more MNK was expressed in the transfected cell lines. This level of expression corresponded well with the intensity of the fluorescent signal observed in the whole cell fluorescence experiments described below.
The intracellular location of MNKm1-3, MNKm4-6, and MNKm1-6 was assessed by confocal immunofluorescence microscopy. All of these mutated proteins localized to the TGN in cells cultured in basal medium (Fig. 3B). When cells were incubated for 3 h in 80 μM copper, some redistribution to the plasma membrane was noticeable in the wt cells and in cell line 114 (MNKm1-3). In high copper concentrations, the labeling of the plasma membrane of these cells intensified and was the predominant signal at 300 μM copper (Fig. 3B). When the 114 and the wt cell lines were incubated for 10, 20, 40, 60, and 120 min in media containing 100 μM copper, there were no noticeable differences in the kinetics of the translocation observed for the two MNK forms (data not shown). In contrast to wt MNK and MNKm1-3, MNKm4-6 and MNKm1-6 remained at the TGN after incubation in high copper concentrations (300 μM). These data demonstrated that the first three MBSs were not essential for the copper-dependent redistribution of MNK, whereas the three MBSs closest to the transport channel were critical.

To further localize the regions critical for relocalization, a construct with MNK mutated in MBS 5 and MBS 6 was created (MNKm5m6). CHO-K1 cells transiently transfected with this construct showed clear perinuclear staining that indicated TGN localization of MNKm5m6. In the presence of elevated copper, MNKm5m6 remained at the TGN, whereas wt MNK relocated to the plasma membrane (Fig. 4). This result showed that MBSs 1–4 were not sufficient for the copper-dependent redistribution of MNK.

To determine whether amino acids other than the MBSs in the N-terminal region play a role in copper-induced trafficking and whether both MBS 5 and MBS 6 or just one of the two were essential, a construct encoding MNK that lacked MBSs 1–4 was generated (MNKΔ1-4) and mutated individually at MBS 5 (MNKΔ1-4m5) and MBS 6 (MNKΔ1-4m6). Immunofluorescence microscopy using an antibody directed against the MNK C terminus showed that in basal media, all three mutant proteins were located at the TGN of transiently transfected CHO-K1 cells. However, when cells were incubated in 200 μM copper for 3 h, an elevated, dispersed fluorescence was evident throughout the cytoplasm and at the plasma membrane (Fig. 5). These results showed that amino acids 8–485 of the MNK N terminus were not necessary for the copper-induced redistribution of the protein; therefore, this region did not contain a critical copper-sensing or copper-responsive targeting signal. In addition, these data indicated that a single MBS located within a maximum distance of 170 amino acids from the membrane channel was sufficient for MNK trafficking.

The ability of the mutated proteins to confer copper resistance was determined by assessing the survival of 114, 115 and 116 cells (expressing MNKm1-3, MNKm4-6, and MNKm1-6, respectively) compared with the parental CHO-K1 cells and CHO-K1 cells expressing wt MNK in increasing copper concentration.

**Fig. 2. Effect of copper on MNK localization in transiently transfected CHO-K1 cells expressing wt MNK and MNK mutated in MBS 1 and MBS 6.** Cells transiently transfected with cDNA encoding MNK mutated in MBS 1 or MBS 6 were cultured for 3 h in normal media (−Cu) or in media containing 200 μM CuCl₂ (+Cu). Cells were then fixed, blocked, and immunolabeled using an affinity-purified N-terminal anti-MNK antibody. wt, wild-type MNK; MNKm1, MNK mutated in MBS 1; MNKm6, MNK mutated in MBS 6. Some untransfected cells are visible, showing the level of background staining.
trations (Fig. 6). The level of copper resistance of cell lines 115 and 116 was similar to that of the parental CHO-K1 cells with an estimated LD$_{50}$ of 60–65 $\mu$M CuCl$_2$ (Fig. 6, B and C). In contrast, the survival of 114 cells and the wt cell line was still evident up to 105 $\mu$M CuCl$_2$, with a LD$_{50}$ of 75 and 105 $\mu$M copper, respectively (Fig. 6A). However, the difference in copper resistance of 114 cells compared with wt cells may be explained by a $\sim$50% reduction in the level of MNK expression.

**Fig. 3.** A, Western blot analysis of stable cell lines expressing wt and mutant MNK. Whole cell protein extracts were prepared from CHO-K1 cells and from stable cell lines expressing wt and mutant MNK; 30 $\mu$g of total protein were separated by 7% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, immunolabeled with an affinity-purified anti-MNK antibody directed against the N terminus, and analyzed by chemiluminescence. CHO-K1, level of endogenous MNK expressed in CHO-K1 cells; wt, CHO-K1 cells expressing wt human MNK; 114, CHO-K1 cells expressing MNK mutated in MBSs 1–3; 115, CHO-K1 cells expressing MNK mutated in MBSs 4–6; 116, CHO-K1 cells expressing MNK mutated in MBSs 1–6. The positions of the molecular mass markers (Bio-Rad) are indicated on the left in kilodaltons (kDa). The arrow indicates the position of MNK at $\sim$178 kDa. B, effect of copper on MNK localization in stable cell lines expressing wt and mutant MNKs. Stable cell lines were incubated in normal media containing the indicated concentrations of copper as CuCl$_2$ for 3 h. Cells were then fixed, blocked, and immunolabeled as described under “Experimental Procedures.” wt, stable cell line expressing wild-type MNK; MNKm1-3, MNKm4-6, and MNKm1-6, stable cell lines 114, 115, and 116, respectively, expressing MNK mutated in MBSs 1–3, MBSs 4–6, and MBSs 1–6, respectively.
in 114 cells (Fig. 3A); therefore, MNKm1-3 may be functionally equivalent to wt MNK.

Taken together, these results showed that: (i) point mutations converting either a single MBS or the first three MBSs from GMXCCXC to GMXSXXX have no effect on the copper-induced intracellular redistribution of MNK as shown with MNKm1, MNKm6, and MNKm1-3; (ii) the protein region encompassing the first four MBSs is not necessary for copper-regulated trafficking; (iii) one MBS within the region comprising MBS 5 to MBS 6 is necessary and sufficient for copper-dependent MNK trafficking; and (iv) the ability of MNK to confer resistance to copper is linked to its ability to undergo copper-induced redistribution.

**DISCUSSION**

Petris et al. (17) previously demonstrated a copper-responsive relocalization of MNK from the TGN to the plasma membrane in copper-resistant CHO-K1 cells. Recently, a copper-dependent redistribution to the cell surface was demonstrated in CHO-K1 cells that expressed human MNK (20). To explain the MNK response to elevated copper levels, it was postulated that the N terminus functioned as a copper-sensing domain in...
which the six MBSs became progressively occupied by copper, causing a conformational change that triggered the redistribution of MNK (17). In this study, we have used an extensive mutagenesis strategy to define the role of the six MBSs in the copper-induced trafficking of MNK.

The mutation of a single MBS did not inhibit the copper-induced relocalization of MNK from the TGN to the plasma membrane, as demonstrated by mutations of MBS 1 and MBS 6 (Fig. 2). The majority of point mutations that have been identified in Menkes patients occur within exons 7–10, which encode MBS 6 and the first four transmembrane helices. All of the point mutations identified within the N terminus were either nonsense mutations or insertion/deletion mutations that are predicted to result in a truncation of the MNK gene product (29). These observations suggest that point mutations that disrupt the function of a single MBS are not likely to cause a disease phenotype and are supported by our findings that six functional MBSs are not necessary for MNK trafficking.

In elevated copper, MNKm1-3 was able to redistribute to the plasma membrane, demonstrating that the first three GMXXCXXC repeats are not essential for the copper-induced trafficking of MNK (Fig. 3B). In contrast to MNKm1-3, MNKm1-6, MNKm4-6, and MNKm5-6 remained predominantly within the perinuclear region, even under very high copper concentrations (300 μM) (Figs. 3B and 4). This result suggested that the MBSs close to the membrane channel were more important for the intracellular trafficking of MNK. Further support for this conclusion was obtained with the deletion constructs containing only MBS 5 and MBS 6 that showed that only one functional MBS close to the channel was necessary and sufficient for MNK trafficking (Fig. 5). These experiments demonstrated that 478 amino acids within the MNK N terminus did not contain a critical targeting sequence and were not required for the copper-dependent exocytic trafficking of MNK, effectively negating the hypothesis that the multiple metal binding sites constitute a relocalization domain.

The ability of the mutated MNK molecules to traffic corresponded well with their ability to confer copper resistance. The parental CHO-K1 cells and cell lines 115 and 116 that overexpressed MNK mutated in MBSs 4–6 and MBSs 1–6, respectively, did not survive in copper levels greater than 75 μM for 7 days. In contrast, the cell lines overexpressing MNKm1-3 and wt MNK (114 and wt cells, respectively) were significantly more resistant to copper (Fig. 6, A–C). This experiment demonstrated that the copper-resistant phenotype was caused by the overexpression of MNK. Therefore, MNK molecules mutated in the first three MBSs were able to effectively efflux copper, indicating that MBSs 1–3 were not essential for both MNK trafficking and copper transport.

Earlier data suggested that MNK constitutively recycled between the TGN and the plasma membrane in cells cultured in low-copper media (17). Copper-induced relocalization of MNK could involve an increase in the rate of exocytosis of MNK from the TGN to the plasma membrane or a reduction in the
rate of endocytosis from the plasma membrane. No published data distinguish between these two possibilities. However, two regions important for its basal location in the TGN have been identified. The first is a 38-amino acid sequence containing transmembrane domain 3, characterized as a candidate region responsible for Golgi retention of MNK (30). In addition, TGN localization has recently been shown to depend on a di-leucine motif, 1487LL1488, within the C-terminal region of MNK (26). Di-leucines are a class of endocytic targeting signals directing plasma membrane proteins into clathrin-coated vesicles, resulting in constitutive internalization of these membrane proteins (31). Based on these observations, one hypothesis to explain the accumulation of MNK at the plasma membrane is that copper retards the internalization of the protein by causing a conformational change that obscures the C-terminal di-leucine motif. This conformational change may occur as a consequence of the binding of copper to MBS 5 or MBS 6. To clarify whether this mechanism is operative, some measurements of the rate of internalization of MNK in the presence and absence of copper will be required.

An alternative model to explain copper-induced relocalization is that conformational changes induced by copper-binding at the N terminus may activate the delivery of copper to the channel and induce the translocation of a copper ion across the cell membrane. Further conformational changes that are coincident with the copper pumping mechanism of MNK may reveal a motif that is recognized by the vesicular sorting machinery, leading to an increase in the number of MNK molecules that are loaded onto vesicles destined for the plasma membrane. In this mechanism, the proximity of the MBS to the transduction channel may be critical either for the direct delivery of copper to the channel or for the acceptance of copper from copper chaperones such as HAH1 (32) that specifically interact with the protein region comprising MBS 5 and MBS 6. This model is supported by several recent experiments from our laboratory that showed different mutations in structurally and functionally distinct regions of MNK, which are predicted to disrupt copper transport activity, inhibited the copper-dependent trafficking of the protein. In addition, Payne et al. (22) have reported that a mutation in a region distinct from the metal binding sites in the closely related Cu-ATPases affected in Wilson disease also abolished copper-induced relocalization (22). These observations suggest that mutations in MNK that disrupt copper transport may also prevent copper-induced relocalization to the plasma membrane.

The redundancy of 478 N-terminal amino acids containing four MBSs implies that as with bacteria and yeast, a Cu-ATPase with only one MBS or two MBSs at the N terminus may be sufficient to maintain some degree of copper homeostasis in mammalian cells. The number of MBSs at the N terminus has increased during evolution: one in bacteria, two in yeast, three in nematodes, and six in mammals. This observation suggests that MNK and WND gained the additional MBSs simply by amplification of the repeated elements. The additional MBSs may have evolved to increase the efficiency of copper scavenging in the cytoplasm or copper ion delivery to the transduction channel in the cells of higher organisms. However, to further clarify the role of the six MBSs in the human copper-transporting ATPases, additional studies involving copper transport activity measurements of the mutant MNK forms will be required.

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