Functional Analysis of the N-terminal CXXC Metal-binding Motifs in the Human Menkes Copper-transporting P-type ATPase Expressed in Cultured Mammalian Cells*

(Received for publication, April 14, 1999, and in revised form, May 21, 1999)

Ilia Voskoboinik†, Daniel Strausak§, Mark Greenough‡, Hilary Brooks‡, Michael Petris§, Suzanne Smith¶, Julian F. Merce,§ and James Camakaris‡‡

From the †Department of Genetics, University of Melbourne, Parkville, Victoria 3052, Australia, the §Centre for Cellular and Molecular Biology, School of Biological and Chemical Sciences, Deakin University, Burwood, Victoria 3125, Australia, and the ‡Radio-Pharmaceutical Division, ANSTO, Lucas Heights Research Laboratories, Lucas Heights, New South Wales 2234, Australia

The Menkes protein (MNK) is a copper-transporting P-type ATPase, which has six highly conserved metal-binding sites, GMTCXXC, at the N terminus. The metal-binding sites may be involved in MNK trafficking and/or copper-transporting activity. In this study, we report the detailed functional analysis in mammalian cells of recombinant human MNK and its mutants with various metal-binding sites altered by site-directed mutagenesis. The results of the study, both in vitro and in vivo, provide evidence that the metal-binding sites of MNK are not essential for the ATP-dependent copper-transporting activity of MNK. Moreover, metal-binding site mutations, which resulted in a loss of ability of MNK to transport copper across membranes, produced a copper hyperaccumulating phenotype. Using an in vitro vesicle assay, we demonstrated that the apparent $K_m$ and $V_{max}$ values for the wild type MNK and its mutants were not significantly different. The results of this study suggest that copper-transporting activity of MNK and its copper-induced relocation to the plasma membrane represent a well coordinated copper homeostasis system. It is proposed that mutations in MNK which alter either its catalytic activity or/and ability to traffic can be the cause of Menkes disease.

The Menkes protein (MNK, ATP7A) is a copper-transporting P-type ATPase (1–3) found in most tissues except the liver. Mutations in the MNK gene cause Menkes disease, a disorder associated with systemic copper deficiency, which is believed to be because of low copper absorption from the small intestine (4). Severe neurodegenerative and connective tissue disorders observed in Menkes patients are thought to be caused by partial or complete loss of catalytic activity of essential copper enzymes (4, 5).

The cDNA-derived amino acid sequence of the MNK protein reveals significant structural similarity with transmembrane P-type ATPases, a common class of cation-transporting transmembrane proteins (6). Among these are heavy metal-transporting Cu$^{2+}$/Cd$^{2+}$ ATPases (7). A unique feature of copper-transporting P-type ATPases is the presence of putative metal-binding site(s) (MBS), GXXCXXC, in the N-terminal region: one in bacteria Enterococcus hirae (CopB), two in yeast Saccharomyces cerevisiae (Ccc2p), three in nematodes Caenorhabditis elegans (8), and six in mammals (MNK and Wilson protein, WND) (9). The GXXCXXC motif is also present in the putative copper chaperones Atox1 and Atx1, which have been proposed to deliver copper to MNK and Ccc2p, respectively, via a ligand exchange mechanism (10).

Several studies have been conducted in an attempt to elucidate the role of MBSs. Lutsenko et al. (11) have demonstrated that the N-terminal domain of MNK binds six atoms of copper per molecule, suggesting each MBS binds one copper. By progressively mutating the MBS of MNK, Payne et al. (12) have recently demonstrated that MBSs 3–6 are required to complement the ΔCCC2 phenotype in S. cerevisiae. In contrast, Iida et al. (13) have shown that only MBS 6 in WND was required to rescue the ΔCCC2 phenotype. Vulpe et al. (14) have provided evidence that the ΔCCC2 phenotype can be complemented by mutant forms of Ccc2p, but at least one MBS was required. In contrast, it has been shown that the mutation of the only MBS in the cadmium-transporting P-type ATPase in Staphylococcus aureus (CaaD) reduced but did not abolish the catalytic activity of the protein, suggesting that the single MBS was not essential for the translocation of cadmium (15).

An important aspect of MNK physiology is that copper regulates the intracellular location of the protein (16). MNK normally resides in the trans-Golgi network (TGN) (16–18), but the elevation of extracellular concentrations of copper results in translocation of MNK from the TGN to the plasma membrane (PM), thus presumably facilitating copper efflux (16). We have proposed, therefore, that MNK can maintain copper homeostasis by means of vesicular trafficking and ATP-dependent copper-transporting activity (16). Strausak et al. (19) demonstrated recently that CXXC to SXXS mutations in MBSs 4–6 or 1–6 abolished the copper-stimulated trafficking of MNK to the PM, whereas mutations in MBSs 1–3 had no effect on the trafficking of MNK compared with the wild-type protein. An important yet unanswered question is whether the processes of MNK trafficking and copper-transporting activity are co-dependent or independent events.

The studies presented in this paper provide the first direct evidence of catalytic activity of human MNK and its variants with mutated MBSs expressed in mammalian cells. Recently we demonstrated that mammalian MNK translocates copper across membranes in vitro, and this is ATP-dependent (20). In
this paper we used a similar in vitro system to demonstrate that MBSSs are not essential for copper-translocating activity of MNK. Moreover, studies using whole cells demonstrated that mutations in MBSSs 4–6 or all six MBSSs resulted in a copper-accumulating phenotype. This phenomenon coincided with the inability of the same mutant proteins to traffic to the PM in response to copper (19). Taken together, our results indicate that MNK trafficking and copper-translocating activity are integral components of intracellular copper homeostasis.

**EXPERIMENTAL PROCEDURES**

*In Vitro Mutagenesis, Transfection, and Cell Culture—*MNK constructs with mutated CXXC to SXXS in MBSSs were produced as described by Strausak et al. (19). The parental CHO-K1 cells were transfected with wild-type human MNK cDNA (117), MNK with MBSSs 1–3 mutated (114), MNK with MBSSs 4–6 mutated (115), MNK with MBSSs 1–6 mutated (116) (Fig. 1), and an empty vector (EV) as control (19). The transfections resulted in varying levels of expression of recombinant proteins. The growth conditions for the transfected cells were as described previously (21).

**Copper Radioisotopes**—The copper radioisotope $^{64}$Cu was produced by Australian Radioisotopes (Lucas Heights, NSW, Australia). The specific activity of $^{64}$Cu varied between 0.2 and 10 Ci/mg copper.

**Isolation of MNK-enriched Membranes and Vesicle Assay of Copper-translocating Activity**—Cells were grown in basali medium until confluent and then homogenized as described earlier (20). The MNK protein-enriched low density membrane fraction (Golgi membrane-enriched) was obtained by using the method described previously (22, 23). The membranes were resuspended in homogenization buffer without EDTA and stored at $-70$ °C. Under these storage conditions, the rates of copper transport were not affected for at least 2 months in the assay described below. Protein concentration of the vesicle preparation was determined using Bio-Rad protein reagent (Bio-Rad Laboratories) (24). The transfections resulted in varying levels of expression of recombinant proteins. The growth conditions for the transfected cells were as described previously (21).

**Copper Accumulation Using Whole Cells**—Experiments on the accumulation and efflux of $^{64}$Cu by whole cells were conducted as described previously (21), and the values were standardized against total cellular protein. The cells were incubated in the presence of 189, 252, or 284 μM CuCl$_2$ for 6 h, or at 63, 126, or 158 μM CuCl$_2$ for 3 days and harvested as described previously (21). Under these conditions, copper did not have a significant effect on cell viability. Protein concentrations were determined using Bio-Rad protein reagent (24). The results are presented as the means of three repeats ± S.D., unless otherwise stated. Analysis of variances (ANOVA) was used, where indicated, as a criterion for significant differences.

**Immunoblotting Analysis**—Vesicles were lysed in 0.2% SDS, and whole cell extracts were prepared as described previously (21). Proteins were resolved on a 4–20% SDS-polyacrylamide gradient gel (Novex, San Diego, CA) and transferred onto a nitrocellulose membrane as described previously (21). MNK was detected using polyclonal rabbit antibodies raised against the polypeptide with the amino acid sequence RNSPLPSLGLIVNYSRASIC within the C-terminal region of MNK (19). MNK was visualized using the enhanced chemiluminescence kit (Roche Molecular Biochemicals). As pure MNK is not available, the relative amount of MNK in purified vesicles from transfected cells was normalized against the level of wild type MNK in cell line 117 by using laser densitometry (Molecular Dynamics, Model 300A).

**RESULTS**

**Vesicle Assays for MNK Copper-translocating Activity**—We have previously described an assay for MNK copper-transport activity using plasma membrane-enriched vesicles isolated from whole cells (20). In contrast, low density Golgi-enriched membrane fractions were utilized in this study, as some mutant MNKs used here did not undergo copper-induced trafficking from the TGN to the PM (19). The copper-translocating activity of MNK was found to be reproducible between independent preparations (Table I).

**Copper-translocating activity of MNK was observed in the presence of ATP, and 50 μM orthovanadate inhibited it to 39 ± 15% (S.D.) of control (no orthovanadate) (Fig. 2). In addition, no reaction was detected in the presence of ADP or the nonhydrolyzable ATP homologue adenosine-5'-[β,γ-methylene]triphosphate (AMP-PCP) (data not shown). The level of ATP-driven copper uptake in vesicles from 117 cells (wild type Menkes) in the reaction buffer supplemented with 1 M sucrose was 60% of the control (no added sucrose) levels. These data indicated that copper translocation was osmotically sensitive and that the association of copper with the vesicles was dependent on their volume, thus suggesting that copper was translocated into the vesicles rather than bound to their surface. There was no ATP-dependent copper transport observed when vesicles were lysed with 0.1% Triton X-100, and copper uptake was temperature-dependent with the rate being reduced by 50% at 22 °C compared with the rate at 37 °C. Dithiothreitol (DTT) was essential for the ATP-dependent translocation of copper, as there was no reaction observed in its absence (data not shown), consistent with our earlier finding suggesting that Cu$^{+}$ is being translocated (20). Overall, these results indicated that copper was actively translocated into the MNK-enriched vesicles (20).

The results shown in Table I indicate that all the MNK mutants tested retained substantial catalytic activity relative to wild-type MNK. The mutant proteins had at least 55% of the wild type activity when standardized for the relative amount of

**TABLE I**

Catalytic activities of the wild-type MNK and its metal binding site mutants

| Cell line | Catalytic activity$^{a}$ | Normalized MNK expression$^{b}$ | Normalized catalytic activity$^{c}$ | Percent residual activity$^{d}$ |
|-----------|-------------------------|---------------------------------|----------------------------------|-------------------------------|
| EV        | $0.028 ± 0.011$         | 0.02                            | NA$^{e}$                         | NA$^{e}$                      |
| 117       | $0.390 ± 0.067$         | 1.0                             | 0.390                            | 100                           |
| 114–1     | $0.347 ± 0.068$         | 1.3                             | 0.267                            | 68                            |
| 114–2     | $0.090 ± 0.044$         | 0.3                             | 0.300                            | 77                            |
| 115       | $0.069 ± 0.035$         | 0.2                             | 0.345                            | 88                            |
| 116–1     | $0.107 ± 0.031$         | 0.5                             | 0.214                            | 55                            |
| 116–2     | $0.082 ± 0.009$         | 0.3                             | 0.273                            | 70                            |

$^{a}$ pmol of Cu/mg of total protein in the presence of 2 μM Cu and 5 mM ATP.

$^{b}$ The levels of expression of MNK mutants relative to the level of MNK in 117 as compared by laser densitometry. The absolute amount of MNK could not be measured as there is no pure MNK available to use as a standard.

$^{c}$ Catalytic activity expressed in pmol of Cu/min/mg of total protein corrected for the relative levels of expression of MNK mutants.

$^{d}$ Catalytic activity expressed as % of activity of 117.

$^{e}$ NA, not applicable.
recombinant MNK expressed in these cells (Fig. 3) providing strong evidence that the MBSs were not essential for copper translocation. The rates of copper translocation by 114 and 116 mutants were inhibited by 50 $\mu$M orthovanadate to 38–62% and 26–69% (S.D.) of control (no orthovanadate) levels, respectively, thus indicating that the mutant MNKs retained a P-type ATPase activity (20). This inhibition, together with the ATP-dependent and ADP-independent catalytic activities, suggests that the copper translocation by mutant MNK was occurring via the same mechanism as wild type MNK, rather than the mutations in MNK resulting in unregulated passage of copper through the ion channel. All the mutants tested showed apparent Michaelis-Menten kinetics with respect to copper (Fig. 4).

We were unable to study the detailed kinetics of the 115 mutant because of the low level of expression of the mutant MNK in these cells (Fig. 3). While apparent $K_m$ values varied insignificantly between different mutants and wild-type MNK (2.8–4 $\mu$M), apparent $V_{max}$ for 116–1 was 2.4-fold lower than for 117 (after standardizing for the amounts of MNK). This is consistent with the partial loss of activity of the 116 mutant (Table I).

The in vitro assay suggested that the mutant MNK molecules retained copper transport activity. However, Strausak et al. (19) have shown that particular N-terminal mutations cause varying degrees of impairment of copper-induced trafficking of MNK. To confirm that the mutant MNK molecules were able to transport copper in vivo and to investigate the consequences of the expression of an active MNK which is unable to traffic, we studied the copper accumulation in CHO cells expressing either normal or mutant MNK constructs.

6-h Copper Accumulation by Whole Cells—Following a 6-h incubation in 189, 252, or 284 $\mu$M copper, 117 cells (wild-type MNK) accumulated significantly less copper than the cells transfected with vector alone (EV) (Fig. 5), consistent with the earlier report by Camakaris et al. (21) and La Fontaine et al. (25) that copper accumulation was reduced and efflux increased in CHO cells overexpressing wild-type MNK. The accumulation of copper by the mutant 114–2, which can traffic to the PM in the presence of elevated copper (19), was significantly lower than in EV cells in the presence of 189 or 252 $\mu$M copper. In this study, the 114–2 clone was used because its level of expression of MNK was comparable with 115 and 116–1 cells (Fig. 3, Table I). Significantly, MNK mutants 115 and 116, whose trafficking is not stimulated by elevated concentrations of extracellular copper (19), accumulated 20 and 30% more copper, respectively, than the EV cells after 6 h in 284 $\mu$M copper (Fig. 5). The low level of expression of the non-trafficking MNK mutant 115, compared with 116, can explain the slightly smaller copper accumulation observed for this mutant at 284 $\mu$M copper (Fig. 5).

72-h Copper Accumulation by Whole Cells—As seen in Fig. 6, there was no significant difference between the levels of copper accumulation by the EV and wild-type MNK overexpressing 117 cells in an experiment where the cells were grown in the
presence of 63, 126 and 158 μM copper thus indicating the copper homeostasis system of the EV cells containing only endogenous MNK was not overloaded with copper. However, the 116–1 mutant cells were unable to maintain copper homeostasis under these conditions, and accumulated twice as much copper as the EV cells after 3 days in 126 and 158 μM copper (Fig. 6). The accumulation of copper by EV cells did not increase significantly at 126 μM compared with 63 μM copper, whereas the 116 mutant accumulated 4-fold more copper at 126 μM compared with 63 μM extracellular copper (Fig. 6). In addition, the 116 mutant cells showed more sensitivity to copper in a colony survival assay when compared with EV (19). We were unable to conduct these studies with the 115 mutant cells because of their slow growth rate, which was the source of experimental inconsistencies.

**DISCUSSION**

The present study provides the first detailed functional analysis of N-terminal putative copper-binding motifs (MBS) of MNK in mammalian cells by studying mutants where these motifs have been altered by site-directed mutagenesis. The overexpression of the normal and mutant MNKs enabled us to analyze the kinetics of MNK-mediated copper translocation using an in vitro vesicle assay. The results have shown that putative MBSs in the N-terminal domain of MNK were not essential for copper-translocating activity of MNK, as all the mutated proteins investigated retained their ATP-dependent copper-translocating activity (Table I, Fig. 4).

The in vitro results (Fig. 5 and 6) are particularly noteworthy and are consistent with the retention of copper transport activity of the mutant MNKs in vitro, but those mutants which have lost the ability to traffic in response to copper (115 and 116) actually lead to enhanced copper accumulation in whole cells relative to the EV control. We suggest that this is a result of these mutant MNK proteins transporting copper into an intracellular compartment, presumably the TGN, from where there is little or no copper-regulated trafficking of MNK to the PM (19), and thus is substantially reducing copper efflux from these cells. The small reduction in 64Cu accumulation in mutant 115 (6 h, 189 μM copper; Fig. 5) may be because of copper efflux occurring as a consequence of a constitutive (copper-independent) recycling pool of catalytically active MNK (16). The low expression of MNK in 115 cells (compared with 116), such a pool would be proportionately higher relative to the pool of catalytically active MNK that does not traffic in response to copper.

The role of putative MBSs in the N terminus of MNK and other copper-transporting ATPases and in particular “the reason” for the six MBSs in the mammalian copper ATPases is not understood. Previous studies utilizing yeast complementation assays have suggested, in contrast to our results, that the MBSs are needed for the copper-transporting activity of MNK, WND, and Ccc2p (Refs. 12–14; see the Introduction). The yeast system involves an indirect measurement of the activity of MNK, WND, and Ccc2p through ability to form the [Fet 3-Cu] complex (12–14) in copper-deficient medium. Mutations of MNK or WND could reduce, but not abolish, their catalytic activities (as reported in this paper), but the reduced activity may be insufficient to complement the ΔCCC2 phenotype. In addition, MBSs may be required to scavenge and concentrate the low amounts of copper present used in the yeast ΔCCC2 complementation assay. It is noteworthy that, using a direct assay on the Cd2+-transporting P-type ATPase CadA in S. aureus, Nuicifora et al. (26) demonstrated that this protein contains only one MBS, and the mutations of cysteines in the sequence GFTCANC reduced but did not abolish the rates of Cd2+ transport (15). Therefore it is clear that the metal-binding sites are required for some of the functions carried out by these enzymes, but our in vitro results show that copper translocation is possible without the N-terminal CXXC motifs. It is not possible to reconcile the results from the different groups at this stage. However, one should also consider the possibility that there may be a number of modes of delivery of copper to the ion channel, one involving the MBSs and another MBS independent, and the various assays are measuring these distinct modes of presentation.

Related to this point is the involvement of copper chaperones, e.g. Atx1 in yeast and Atox1 in humans, in the delivery of copper to the enzymes. The inability of MBS mutants 115 and 116 to traffic, while being catalytically active suggests that one of the roles of MBSs is copper binding, possibly via the [Atox1-Cu+] complex, followed by the recognition of copper-loaded MNK by the vesicle-assembling system. In the in vitro system described here, it is possible DTT functioned not only as

---

*2 I. Voskoboinik, M. Greenough, and J. Camakaris, unpublished observations.
a reducing agent for copper, but also as a copper chaperone as it can bind copper (27) in a complex similar to the CXXC motif (the structure of DTT is HS-CH₂(CHOH)₃CH₂SH). One can argue that there is a potential for copper to bypass MBSs and be delivered to the channel directly, especially in MBS mutants and this may be the mechanism in cells exposed to high copper. As copper translocation for all the MNK proteins tested was stimulated by ATP and inhibited by orthovanadate, this direct delivery still has the properties of a P-type ATPase transport system.

In conclusion, our studies provide biochemical and physiological evidence that MBSs of human MNK are not important for its catalytic activity, but the presence of at least MBSs 4, 5, and 6, which may function as sensors for copper, appears to be essential for copper-regulated trafficking (19). Copper-translocating activity and copper-stimulated trafficking of MNK together represent a finely tuned regulated intracellular copper homeostasis system. The current findings suggest that MNK can perform its physiological role in detoxification of copper and in the absorption of copper from small intestine into the blood stream only when the both functions are intact. It can be predicted that mutations, which result in the loss of catalytic activity and/or copper-stimulated trafficking ability of MNK, would result in copper accumulation and, potentially, Menkes disease.

REFERENCES

1. Vulpe, C., Levinson, B., Whitney, S., Packman, S., and Gitschier, J. (1993) Nat. Genet. 3, 7–13
2. Chelly, J., Tumer, Z., Tonnesen, T., Petterson, A., Ishikawa Brush, Y., Tommerup, N., Horn, N., and Monaco, A. P. (1993) Nat. Genet. 3, 14–19
3. Mercer, J. F. B., Livingston, J., Hall, B., Paynter, J. A., Begy, C., Chandrasekharappa, S., Lockhart, P., Grimes, A., Bhave, M., Siemieniak, D., and Glover, T. W. (1993) Nat. Genet. 3, 20–25
4. Danks, D. M. (1995) in The Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. V., and Valle, D., eds), pp. 2211–2235, McGraw-Hill, New York
5. Mercer, J. F. B., and Camakaris, J. (1997) in Metal Ions in Gene Regulation (Silver, S., and Walden, W., eds), pp. 250–276, Chapman and Hall, New York
6. Soloz, M., Odermatt, A., and Krapf, R. (1994) FEBS Lett. 346, 44–47
7. Moller, J. V., Juul, B., and le Maire, M. (1996) Biochim. Biophys. Acta 1286, 1–51
8. Sambongi, Y., Wakabayashi, T., Yoshimizu, T., Omote, H., Oka, T., and Futai, M. (1997) J. Biochem (Tokyo) 121, 1169–1175
9. Koch, K. A., O Pena, M. M., and Thiele, D. J. (1997) Chem. Biol. 4, 549–560
10. Pufahl, R. A., Singer, C. P., Pearson, K. L., Lin, S.-J., Schmidt, P., Cizewski-Culotta, V., Penner-Hahn, J. E., and O’Halloran, T. V. (1997) Science 278, 853–856
11. Lutsenko, S., Petrokhin, K., Cooper, M. J., Gilliam, C. T., and Kaplan, J. H. (1997) J. Biol. Chem. 272, 18939–18944
12. Payne, A. S., and Gitlin, J. D. (1998) J. Biol. Chem. 273, 3765–3770
13. Iida, M., Terada, K., Sambongi, Y., Wakabayashi, T., Miura, N., Koyama, K., Futai, M., and Sugiyama, T. (1998) FEBS Lett. 426, 281–285
14. Vulpe, C., Yuan, D., Ibon, V., and Gitschier, J. (1997) in Copper and Zinc Receptors in Signalling, Trafficking and Disease, pp. 35, ASBMB, Granlibakken, Lake Tahoe, CA
15. Konings, W. N., Kaback, H. R., and Lokkema, J. S. (1996) Transport Processes in Eukaryotic and Prokaryotic Organisms pp. 3–4, Elsevier Science Publishers B.V., Amsterdam
16. Petris, M. J., Mercer, J. F., Culvenor, J. G., Lockhart, P., Gleeson, P. A., and Camakaris, J. (1996) EMBO J. 15, 6084–6095
17. Yamaguchi, Y., Heiny, M. E., Suzuki, M., and Gitlin, J. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14030–14035
18. Dierick, H. A., Adam, A. N., Escara-Wilke, J. F., and Glover, T. W. (1997) Hum. Mol. Genet. 6, 409–416
19. Strausak, D., La Fontaine, S., Hill, J., Firth, S. D., Lockhart, P. J., and Mercer, J. F. (1999) J. Biol. Chem. 274, 11170–11177
20. Voskoboinik, I., Brooks, H., Smith, S., Shen, P., and Camakaris, J. (1998) FEBS Lett. 435, 178–182
21. Camakaris, J., Petris, M. J., Bailey, L., Shen, P., Lockhart, P., Glover, T. W., Barcroft, C., Patton, J., and Mercer, J. F. (1995) Hum. Mol. Genet. 4, 2117–2123
22. Simpson, I. A., Yver, D. R., Hissin, P. J., Wardzala, L. J., Karnieli, E., Salans, L. B., and Cushman, S. W. (1991) An. J. Physiol. 260, C570–C580
23. Bradford, M. (1976) Anal. Biochem. 72, 248–254
24. La Fontaine, S., Firth, S. D., Lockhart, P. J., Brooks, H., Patton, R. G., Camakaris, J., and Mercer, J. F. (1998) Hum. Mol. Genet. 7, 1293–1300
25. Nucifora, G., Chu, L., Misra, T. K., and Silver, S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3544–3548
26. Kachur, A. V., Held, K. D., Koch, C. J., and Biaglow, J. E. (1997) Radiat. Res. 147, 409–415