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Menkes Copper-Translocating P-type ATPase (ATP7A): Biochemical and Cell Biology Properties, and Role in Menkes Disease

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The Menkes copper-translocating P-type ATPase (ATP7A; MNK) is a ubiquitous protein that regulates the absorption of copper in the gastrointestinal tract. Inside cells the protein has a dual function: it delivers copper to cuproenzymes in the Golgi compartment and effluxes excess copper. The latter property is achieved through copper-dependent vesicular trafficking of the Menkes protein to the plasma membrane of the cell. The trafficking mechanism and catalytic activity combine to facilitate absorption and intercellular transport of copper. The mechanism of catalysis and copper-dependent trafficking of the Menkes protein are the subjects of this review. Menkes disease, a systemic copper deficiency disorder, is caused by mutations in the gene encoding the Menkes protein. The effect of these mutations on the catalytic cycle and the cell biology of the Menkes protein, as well as predictions of the effect of particular mutant MNKs on observed Menkes disease symptoms will also be discussed.

KEY WORDS: Menkes protein; Menkes disease; copper; P-type ATPase; trafficking.

INTRODUCTION

Copper is an essential trace metal, whose redox properties are utilized by numerous enzymes catalyzing redox transformations of their substrates (Linder and Hazegh Azam, 1996). Systemic copper deficiency can cause reduction in the activity of these vital enzymes, while copper toxicity is manifested in intracellular oxidative stress, which leads to lipid peroxidation, protein cleavage, enzyme inhibition, and DNA damage. Potential severe effects of copper imbalance on cell and organism physiology have resulted in the evolution of copper homeostasis mechanisms, which would allow regulated high affinity uptake of copper, its specific delivery to cuproenzymes via protein–protein interactions, and removal of excess copper through intracellular sequestration and/or efflux from the cell (Camakaris *et al.*, 1999). Therefore the intracellular concentration of highly reactive copper is maintained at extremely low levels by such mechanisms (Rae *et al.*, 1999). The breakdown of any of the above mechanisms can lead to detrimental effects. In humans this is associated with a number of pathological conditions, such as the inherited disorders, Menkes and Wilson diseases, and neurodegenerative disorders such as amyotrophic lateral sclerosis, Creutzfeldt-Jacob and Alzheimer's diseases (Camakaris *et al.*, 1999). Chronic marginal copper deficiency may be important in the aetiology of cardiovascular disease and osteoporosis (Saari, 2000).

The Menkes protein (MNK; ATP7A), which is a copper-translocating P-type ATPase, is one of the key elements of the copper homeostasis machinery in mammals (Camakaris *et al.*, 1999). It is found in most tissues, except the liver, where a homologous copper ATPase, the Wilson protein (WND; ATP7B), is expressed (Paynter, *et al.*, 1994; Schilsky, 1994). MNK first came into prominence when mutations in the *ATP7A* gene were found to cause an inherited X-linked disorder associated with systemic copper deficiency, Menkes disease (Chelly *et al.*, 1993; Mercer *et al.*, 1993; Vulpe *et al.*, 1993). The amino acid sequence prediction analysis of the gene revealed striking structural similarity between

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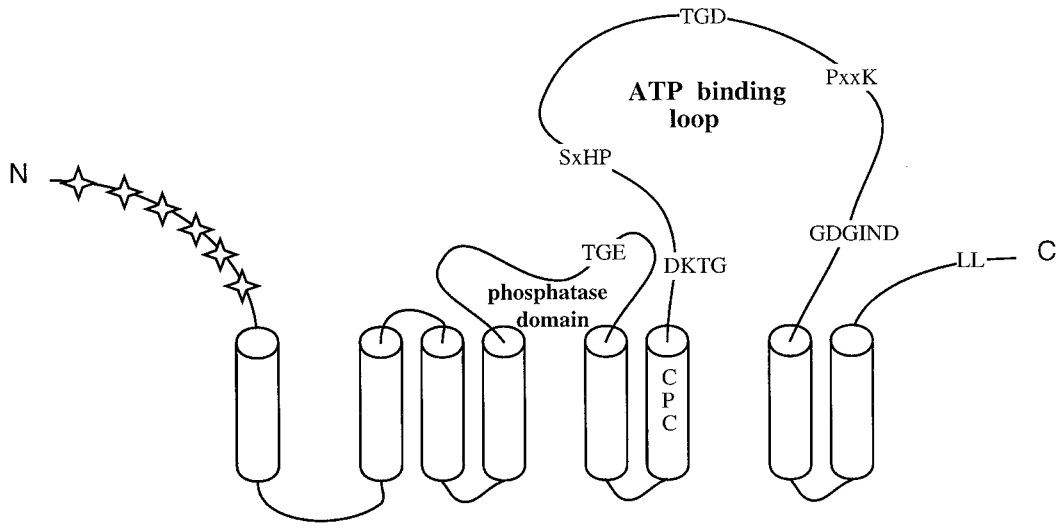

Fig. 1. Schematic structure of the Menkes copper-translocating P-type ATPase. Designated with star symbol are six N-terminal putative metal-binding sites; conserved—TGE—motif in the phosphatase domain, phosphorylation—DKTG—motif, proposed ATP-binding motifs—SxHP—,—TGD—, PxxK, and the "hinge" motif; C-terminal di-leucine (-LL) internalization motif.

MNK and bacterial heavy metal P-type ATPases. This together with the copper-related aetiology of Menkes disease has lead to the hypothesis that MNK is a copper P-type ATPase (Chelly *et al.*, 1993; Mercer *et al.*, 1993; Vulpe *et al.*, 1993). However, it is only recently that biochemical evidence was provided for copper-transporting activity of mammalian Cu-ATPases, MNK, and WND, thus "officially" placing these two proteins in the family of P-type ATPases Group IB, together with bacterial heavy metal transporters (Tsivkovskii *et al.*, 2002; Voskoboinik *et al.*, 1998, 2000a,b). A unique feature of mammalian Cu-ATPases is their ability to undergo copper-dependent vesicular trafficking between the *trans*-Golgi network (TGN) and the plasma membrane (PM) (Petris *et al.*, 1996).

This field has advanced quickly through elegant work from a number of laboratories in the short period since *ATP7A* was cloned. Here we will focus on the catalytic and cell biology properties of MNK, and implications of their disturbance in Menkes disease, with particular emphasis on contributions from our laboratory.

CATALYTIC FUNCTION OF MNK

The predicted amino acid sequence analysis of the *MNK* (*ATP7A*) gene has revealed motifs conserved among non-heavy metal P-type ATPases, e.g., the phosphorylation—DKTG—motif, the phosphatase—TGE—motif, and the Mg^{2+}-binding ("hinge") motif—GDGIND—. In addition, MNK appeared to contain motifs common for bacterial heavy metal P-type ATPases, e.g., the N-terminal metal-binding sites (MBSs)—GMxCxxC—(six repeats of these are in MNK and WND) and the cation transduction—CPC—motif in the transmembrane domain 6 (Fig. 1).

We designed a direct biochemical assay for catalytic activity of mammalian Cu- ATPases, MNK, and WND—the membrane vesicle ^{64}Cu transport assay using various subcellular membrane fractions of cultured mammalian cells. Catalytic activity studies have shown that both MNK and WND require reduced copper, Cu(I), in order to translocate it in an ATP-dependent manner. On that basis we have proposed that Cu(I) is the substrate for human copper ATPases (Voskoboinik *et al.*, 1998, 2001a). However, whether copper undergoes oxidation to Cu(II) prior to release from the channel or released in the lumen as Cu(I) is unknown. In favor of Cu(I) oxidation is that the environment in the lumen of the Golgi, where copper is translocated by MNK, is significantly more oxidizing than in the cytosol (Fig. 2). In addition, the oxidation of Cu(I) in the transmembrane cation channel is an appealing chemical mechanism for the release of, probably, thiolated copper from a putative high affinity binding site in the channel to the lumen, since the oxidation of Cu(I) to Cu(II) would facilitate its transfer to lower affinity copper-binding sites followed by hydration and subsequent release from the channel. Furthermore, our studies indicated that the lysis of copper-loaded vesicles during the ^{64}Cu translocation assay resulted in the release of all copper accumulated in vesicles, which may suggest the presence of

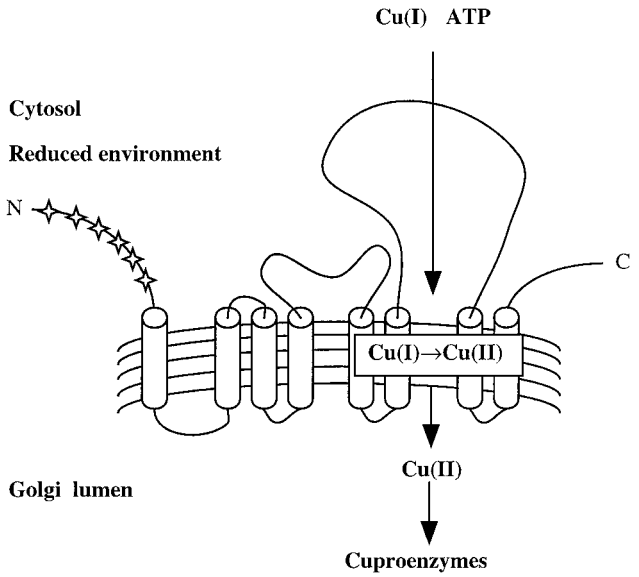

Fig. 2. Proposed redox mechanism of copper translocation and release by the Menkes P-type ATPase.

non-protein-bound (possibly hydrated) Cu(II) rather than Cu(I) which would more likely be protein-bound.

Since no other Cu-transporting systems were detected in vesicles from MNK-overexpressing cells used for the catalytic studies, we assumed that all ATP-dependent copper translocation was due to MNK (Voskoboinik *et al.*, 1998, 1999). The correction of the apparent V_{max} for the absolute amount of MNK using quantitative Western blots with the purified N-terminus of MNK suggested a value of $\approx$0.15 μmol Cu/mg/min (our unpublished observations), which is similar to the value determined for bacterial Cu-ATPases (Bissig *et al.*, 2001), but significantly lower than the activities reported for other P-type ATPase, ($\approx$1–5 μmol/mg/min). The K_m of 3–5 μM copper is an apparent value due to a number of copper-binding components in the reaction buffer (histidine and DTT) and in partially purified membrane vesicles (Voskoboinik *et al.*, 1998, 1999; 2001a,b). Our estimates predict the actual K_m value for Cu(I) at a low nanomolar range. At the same time, the apparent K_m value for ATP was estimated to be $\approx$10–15 μM (Voskoboinik *et al.*, 2001). Given the presumed presence of other ATPases in the preparation it is expected that the "actual" value is even lower, which makes it comparable to the K_m's for other P-type ATPases, commonly 1–5 μM (Daly *et al.*, 1996; Mollman and Pleasure, 1980).

Recently we utilized an acyl-phosphorylation assay for MNK which allowed a more detailed analysis of its catalytic cycle (Voskoboinik *et al.*, 2001a). The rates of MNK acyl-phosphate formation and the turnover were similar to those of other P-type ATPases at $0 + 2^{\circ}C$, approximately 20 s (Voskoboinik *et al.*, 2001b). Interestingly, the labelling with γ^{-32}P-ATP was time-dependent for at least another minute, but the labelled product could not turn over (our unpublished observations). Similar experiments with the WND protein revealed that the acyl-phosphate formed after 20 s of labelling with γ^{-32}P-ATP could not turnover in the presence of 1 mM ATP even after 5 min. Moreover, the toxic milk mouse mutant of WND also appeared to be labelled as fast as the wild-type protein (our unpublished observations), despite the lack of ^{64}Cu-translocating catalytic activity (Voskoboinik, *et al.*, 2001a). A similar mutant in MNK could not be acyl-phosphorylated, which suggested that the mutation disrupted a component of the cation channel (Voskoboinik *et al.*, 2001b). These results suggest that the observed WND labelling was either acyl-phosphate-independent or was masked by a copper-dependent autophosphorylation or a protein kinase-dependent process (discussed below).

The acyl-phosphorylation studies revealed the sequence of events in the catalytic cycle of MNK which is similar to other P-type ATPases (Fig. 3) (Voskoboinik *et al.*, 2001b). The evidence for such a mechanism came from the analysis of catalytic mutants of MNK, which

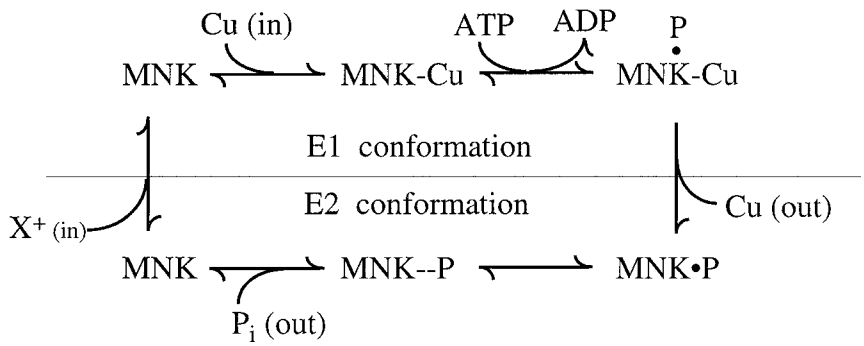

Fig. 3. Proposed model for the catalytic cycle of the Menkes P-type ATPase.

suggested that the binding of copper within the transmembrane channel, whose composition has yet to be fully understood, precedes the binding of ATP and subsequent acyl-phosphorylation of MNK (Voskoboinik, *et al.*, 2001b). Acyl-phosphorylation appeared to be reversible in the presence of ADP and could be inhibited by orthovanadate, a classical inhibitor for P-type ATPases. However, the inhibition occurred at higher concentrations than those reported for non-heavy metal P-type ATPases (Voskoboinik *et al.*, 1998; 2001b). This may explain the marginal level of acyl-phosphorylation by inorganic phosphate, which was also reported for some other heavy metal ATPases (Tsai and Linet, 1993). Overall, the analysis of acyl-phosphorylation of MNK suggested the protein was undergoing conformational changes as a part of the catalytic cycle, where the protein was present in the E1- and E2-like states. The fact that relatively high concentrations of orthovanadate were required for the inhibition of acyl-phosphorylation suggested that the protein was present predominantly in the E1 state, which is characterized by high affinity copper binding (Voskoboinik *et al.*, 2001b). This is probably because of the binding of the cation to the high affinity cytosolic N-terminal MBSs, since their mutation increased the sensitivity of the protein to orthovanadate (see below).

ATP7A has six putative MBSs in the N-terminal domain with a conserved repeat sequence motif GMxCxxC. The role of these sites has been subject to much debate and controversy. Indirect studies suggested some sites were important for copper transport, while others appeared to be redundant (Forbes *et al.*, 1999; Lutsenko *et al.*, 1997; Payne and Gitlin 1998). We utilized direct biochemical assays to clarify the role of the MBSs in catalysis. Through catalytic studies we have shown that the mutation of all six MBSs did not abolish the catalytic activity of MNK, indicating these motifs were not essential for copper translocation (Voskoboinik *et al.*, 1999, 2001b). These findings were confirmed later by other groups working on bacterial heavy metal ATPases (Fan *et al.*, 2001; Mitra and Sharma, 2001). Further analysis revealed that the binding of copper to the MBSs increased the affinity of MNK for copper translocation, probably through intramolecular changes which could involve the interaction between the MBSs and the cytosolic ATP-binding domain of MNK (Tsivkovskii *et al.*, 2001; Voskoboinik *et al.*, 2001). Our results suggest that while the MBSs are not essential for copper translocation *per se*, they may play a regulatory role when copper levels are low. These findings were also interesting with respect to the sensitivity of a commonly used assay for MNK and WND function, based on genetic complementation of the yeast copper P-type ATPase, Ccc2. This assay is carried out utilizing copper and iron-depleted media (Fu *et al.*, 1995; Yuan *et al.*, 1995, 1997). Considerable discrepancies between the effects of mutations on the function of mammalian Cu-ATPases were observed by various research groups using that assay system (Iida *et al.*, 1998; Forbes *et al.*, 1999). Our data suggest that under copper/iron depleted conditions, MNK mutants that have reduced affinity for copper, yet are catalytically active, are unable to complement the activity of its yeast counterpart. The Ccc2 complementation assay appears to be reliable under conditions where the introduced mutation affects the ATP-related function of the protein rather than its affinity for copper.

Another important observation in relation to the role of the MBSs was that these sites were not responsible for the cation specificity of MNK since neither cadmium, zinc, nor mercury could induce the acyl-phosphorylation of MNK, despite their ability to bind to the MBSs (Voskoboinik *et al.*, 2001). This was supported further by recent studies where a chimeric molecule consisting of the N-terminal MBSs of MNK was fused to the catalytic (post-N-terminal) part of bacterial zinc P-type ATPase, ZntA (Voskoboinik and Haltia, unpublished observations). This chimeric molecule had negligible affinity for copper, whereas it could transport both cadmium and zinc (Fig. 4). These findings together suggest that the cation specificity of heavy metal ATPases is primarily determined by the composition of the respective cation channels, while the N-terminal MBSs may function as sensors of heavy metals in the cell, and regulate the catalytic activity, probably through conformational changes induced by the binding of heavy metals.

Understanding the catalytic mechanism and structure–function analysis of MNK is important with respect to the diagnosis and potential therapy of Menkes disease, which presents with various degrees of severity, e.g., the classical severe disorder with profound neurological and connective tissue defects (causing death usually before the age of 3 years), the treated form, and mild Menkes disease, and occipital horn syndrome which is a connective tissue disorder (Danks, 1995). The complete loss of function of MNK because of premature truncation, which often occurs before the first transmembrane domain thus deleting all the catalytic components (Tumer *et al.*, 1997), is predicted to invariably cause classical Menkes disease. In the case of missense or some splice mutations, there is no clear evidence of how much of the original catalytic activity of MNK needs to be preserved to permit absorption of sufficient amounts of intestinal or parenterally administered copper and its delivery to cuproenzymes.

Several Menkes patient missense mutations have been identified, and our existing, though very limited,

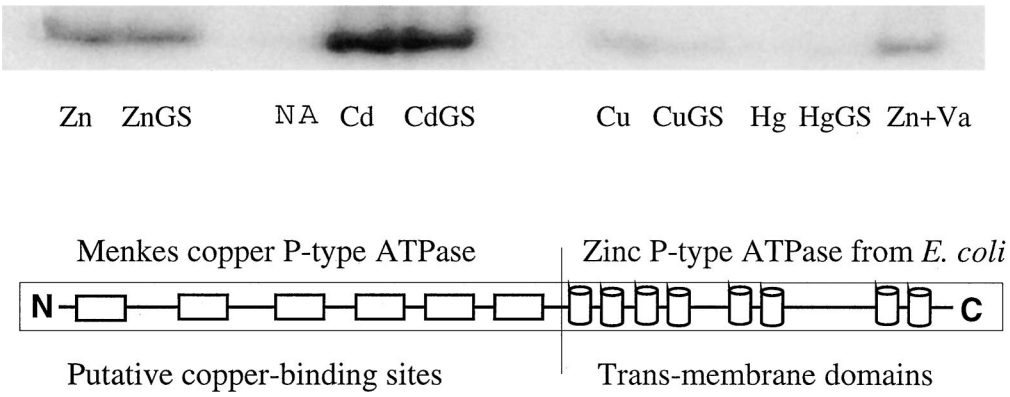

Fig. 4. Acyl-phosphorylation of the chimeric protein consisting of the cytosolic N-terminal domain of MNK fused to the bacterial zinc P-type ATPase, ZntA (lacking its N-terminal cytosolic region). The reaction was conducted as described (Voskoboinik *et al.*, 2001b) in the presence of 5 μM Zn(II), Cd(II), 0.5 μM Hg(II), or the same concentrations of their glutathionates designated as ZnGS, CdGS, CuGS, and HgGS. The inhibition of Zn-dependent acyl-phosphorylation by sodium orthovanadate was conducted in the presence of 1 mM of the inhibitor; NA – no heavy metal cation added.

knowledge of the structure-function of MNK should allow some predictions of the effect of these mutations on catalysis. The disease-causing mutations are commonly represented by nonconservative amino acid substitutions within highly conserved motifs or amino acids (Tumer *et al.*, 1997). Some of these mutations are likely to affect protein structure, while others may influence the catalytic mechanism. For example, Gly727Arg and SerIle1344/1345ArgPhe substitutions caused Menkes disease even though these regions are unlikely to represent any critical catalytic domain (Gu *et al.*, 2001). At the same time, such mutations as C1000R and L1006P are within the essential transmembrane domain 6, which contains the highly conserved ^{1000}CPC motif implicated in cation transduction (Tumer *et al.*, 1999). The latter substitution is likely to disrupt the structure and, hence, the normal function of that domain, while the substitution of the cysteine residue is likely to abolish transmembrane binding of copper, as shown for homologous heavy metal ATPases (Bissig *et al.*, 2001; Forbes and Cox, 2000). Another mutation associated with Menkes disease was the frame-shift leading to the termination of protein at Gln 1288, which abolished the Mg^{2+}-binding region ("hinge" domain) and critical transmembrane domains 7 and 8 (Seidel *et al.*, 2001).

Importantly, a splice mutation involving the +6 position of intron 6 within a copper-binding domain, which was shown to cause Menkes disease, had been detected in a patient with occipital horn syndrome, a mild form of Menkes disease associated, predominantly, with connective tissue abnormalities. Detailed analysis revealed very small amounts of the normal transcript (2–5% of the normal level), which appeared to be sufficient to allow some alimentary copper absorption into the blood and its delivery, through the blood–brain barrier, to the brain (Moller *et al.*, 2000). However, that level of MNK was apparently lower than that required for maturation of lysyl oxidase in the TGN, thus resulting in connective tissue defects, typical of occipital horn syndrome. Laboratory tests, using ^{64}Cu efflux from that patient's fibroblasts, also revealed an intermediate phenotype, where some copper (26%) was shown to be effluxed from the cells, compared to almost 95% retention commnly observed in the Menkes disease patient (Seidel *et al.*, 2001).

In one of the cases of treated Menkes disease, a small deletion between the noncritical amino acids 624 and 649 resulted in two cases of classical Menkes disease. However, the third patient from the same family, who was given parenteral copper replacement therapy from the age of 8 days, had age-appropriate development, suggesting the mutant MNK retained some catalytic activity that allowed copper supply to the brain (Kaler *et al.*, 1996). It appears that very early parenteral copper treatment prevented severe neurological defects associated with Menkes disease. The development of classical Menkes disease in the other two patients suggested clearly that the enzyme activity was insufficient to allow absorption of essential amounts of copper at a very early stage of postnatal development (Kaler, 1998; Kaler *et al.*, 1996).

In essence, on the basis of the clinical studies, it was proposed that as little as 2–5% of functional MNK in the cell is sufficient to at least reduce the severity of Menkes disease (Moller *et al.*, 2000). This agrees with normal hepatocyte transplantation in the liver of Long-Evans Cinnamon rats affected by Wilson disease, where the implantation of as little as 10% normal hepatocytes was sufficient to "cure" the disease (Irani, *et al.*, 2001; Yoshida

et al., 1996). In addition, very early therapy of Menkes patients may be successful in the cases of existence of some functional protein or should the disease-causing mutations occur in catalytically noncritical regions of MNK (Kaler, 1998; Kaler *et al.*, 1996).

MNK TRAFFICKING

Early evidence on ^{64}Cu turnover from Menkes disease patient fibroblasts suggested a defect in copper efflux (Camakaris *et al.*, 1980). Major evidence on the role of MNK in the transport of copper came from mammalian cells, cultured Chinese hamster ovary cells (CHO), which had been selected for copper resistance and found to have *MNK* gene amplification and, consequently, elevated levels of endogenous MNK protein (Camakaris *et al.*, 1995). These cells were not only resistant to elevated levels of copper but also effluxed at a much higher rate than parental cells expressing basal levels of MNK (Camakaris *et al.*, 1995). It was reasonable to predict that MNK would be localized to the PM. However, detailed analyses, by immunofluorescence and electron microscopy, provided an initially unexpected result of MNK localization in the TGN under basal conditions (Petris *et al.*, 1996). Although this observation was consistent with the essential role of MNK in supplying copper-dependent enzymes in the secretory pathway, e.g., lysyl oxidase, it was in apparent conflict with the MNK-assisted rapid efflux of copper from the cell. A major advance in understanding the physiological role of MNK was made in our laboratory when intracellular localization of MNK was investigated under elevated copper concentrations, and the protein was found to traffic from the TGN to the PM; MNK endocytosed back to the TGN once extracellular copper concentrations subsided to normal physiological levels (Petris *et al.*, 1996). In addition, the protein was continuously recycling between the TGN and the PM under basal conditions (Petris and Mercer, 1999). The discovery of MNK trafficking has provided an explanation for its dual role in copper homeostasis, whereby copper can be delivered to cuproenzymes in the TGN at basal levels and effluxed from the PM at elevated Cu levels. Under these circumstances there would appear to be no physiological requirement for two Cu-ATPases in the cell, which is usually the case in lower organisms. Thus in yeast, the primary function of the MNK homologue, Ccc2, is the delivery of copper to cuproenzymes in the Golgi apparatus (Yuan *et al.*, 1997), but it appears to have no role in copper efflux from the cell. However, another Cu-ATPases, Pcal, appears to be involved in copper efflux from yeast (Rad *et al.*, 1994; Shiraishi *et al.*, 2000).

Copper-dependent steady-state relocalization of MNK from the TGN to the PM could potentially be achieved through stimulation of rates of exocytosis to the PM and/or inhibition of rates of endocytosis to the TGN. Quantitative studies are needed to clarify which of these processes take place. Although the exact mechanism of MNK trafficking has yet to be characterized, some aspects are already identified. Thus the retention of MNK at the TGN appears to be controlled by transmembrane domain 3, while the C-terminal dileucine motif ($^{1482/1483}$LL) appears to be responsible for MNK internalization from the PM, thus suggesting the involvement of clathrin-mediated endocytosis (Francis *et al.*, 1998, 1999; Petris *et al.*, 1998). The N-terminal MBSs, whose amplification is observed in evolutionary time from bacteria (one or two MBSs) to eukaryotes (up to six MBSs in humans), also appear to play an important role in sensing elevated intracellular copper concentrations (Goodyer *et al.*, 1999; Strausak *et al.*, 1999). Detailed studies on the role of MBSs in trafficking have shown that only the MBSs 5 and 6 are important for copper-stimulated relocalization to the PM (Strausak *et al.*, 1999). In addition, recent studies have shown that MNK trafficking is dependent on the conformational state of the protein during the catalytic cycle. Thus constitutively acyl-phosphorylated MNK appeared to be retained at the PM, which may indicate the copper-dependent and catalytic cycle-dependent modulation of affinity of MNK to adapter proteins in the TGN and PM (Petris *et al.*, 2002).

Interestingly, the copper chaperone for MNK, Atox1, was demonstrated to deliver copper only to MBSs 1–4, but not to MBS 5 and 6 which are critical for Cu-stimulated exocytosis of MNK (Larin *et al.*, 1999; Strausak *et al.*, 1999). At the same time, Atox1 was shown, using the gene knock-out mouse model, to play a vital role in basal copper absorption, which was probably regulated through the interaction with MNK. However, copper supplementation allowed bypassing of the chaperone and delivery of copper to other organs (Hamza *et al.*, 2001). Two hypotheses on the role of MBSs in copper homeostasis seem to emerge from these findings: (1) Atox1 delivers copper to MBSs 1–4, which results in conformational changes in the N-terminus of MNK leading to the transfer of copper from MBSs 1–4 to MBSs 5 and/or 6. As a result, MNK traffics to the PM. The sensing function of MBSs may be that a certain number of MBSs need to be saturated with copper from Atox1 before the cooperative transfer between the MBSs takes place and the protein starts trafficking. (2) Copper binding to MBSs 5 and/or 6 occurs only after the intracellular concentration of copper reaches such a level that Atox1 is saturated with copper and the delivery of excess copper to MBSs 5 and 6 occurs through some other, possibly nospecific, pathway. That sensing of

elevated copper induces MNK trafficking to the PM where it effluxes copper from the cell. Regardless of which mechanism controls the behavior of MNK, the protein appears to be well equipped to perform its physiological roles in various subcellular compartments.

While the dual function of MNK is "economically" beneficial, there is a potential for a specific impact on copper homeostasis, should the cell biology of MNK be impaired as a result of mutations. Thus a recently identified frame-shift mutation in exon 23 of the *ATP7A* gene results in the premature truncation of the C-terminal end of the MNK protein, including the critical LL1482/1483 (Dagenais *et al.*, 2001). The truncated protein is predicted to localize constitutively at the PM, as was observed in the case of the LL1482/1483AA mutant of MNK, which in our *in vitro* studies appeared to be catalytically active and could confer copper resistance through normal copper efflux (Petris *et al.*, 1998). However, while the localization at the PM would allow for copper absorption to occur, one would predict that the needs of cuproenzymes of the secretory pathway for copper may not be fulfilled. Indeed, the patient with the above truncation mutation in the *MNK* gene only had symptoms of occipital horn syndrome (Dagenais *et al.*, 2001). These symptoms suggested that the function of lysyl oxidase was impaired, most likely because of mislocalization of MNK from the TGN, which significantly reduced the delivery of copper to lysyl oxidase causing connective tissue abnormalities. At the same time, the localization of mutant MNK at the PM would allow sufficient intestinal copper absorption and, hence, no significant neurological defects were observed in the patient.

POTENTIAL MECHANISMS OF MNK REGULATION

Recently published studies on WND suggested that protein kinase-dependent phosphorylation of MNK may be important for the regulation of its function (Vanderwerf *et al.*, 2001). Moreover, recently we also observed protein kinase-dependent phosphorylation of MNK in cultured cells (our unpublished observations; manuscript in preparation). Thus MNK was phosphorylated both constitutively (in copper-depleted media) and in a copper-stimulated manner. Interestingly, a significant increase in phosphorylation was observed at submicromolar concentrations of copper in the culture media.

This feature of MNK may represent a novel signalling pathway of regulation of its function in response to copper. So far no copper-responsive mechanisms of MNK regulation were found at the transcriptional level as occurs in lower organisms for Cu-ATPases, where, for example, gene expression of bacterial copper and zinc P-type ATPases is regulated by copper and zinc respectively (Rensing *et al.*, 1997, 2000). Interestingly, copper treatment of cultured mammalian cells had no apparent impact on the protein turnover or the level of the *MNK* gene expression, but the selection of copper-resistant clones has resulted in gene amplification, rather than isolating regulatory mutants (Camakaris *et al.*, 1995; Petris *et al.*, 1996). Recent studies on the promoter region of WND identified metal-responsive elements in the promoter region, but these were required only for basal expression of the WND gene (Oh *et al.*, 1999, 2002).

Mammalian copper ATPases have evolved regulatory mechanisms that relate to copper controlling their subcellular localization rather than influencing their transcription or translation control mechanisms. One of the major reasons, in our opinion, for this "apparent" simplification of the ATPase system in multicellular organisms, which utilize one transporter in various subcellular compartments, is that each cell type or tissue in differentiated organisms functions as a "barrier" for nutrients, including copper. As a result, most of the tissues would not be exposed to high concentrations of copper. In addition, the normal diet of mammals is not copper-rich and not subject to large and sudden fluctuations in copper levels as would occur for unicellular organisms in a natural environment. Therefore, copper ATPases evolved to utilize rather small amounts of environmental copper efficiently by unidirectional absorption across the basolateral membrane of intestinal cells and reabsorption in polarized epithelial cells of kidneys. Other cell types receive copper from the blood through endothelial cells of capillaries and then pass it on to the underlying tissues or across the blood–brain barrier. This can efficiently be achieved by copper-regulated MNK trafficking to the PM of these cells (basolateral membrane in enterocytes), while under "basal" conditions MNK functions to bind sufficient copper and deliver it to cuproenzymes in the TGN. In contrast, it is reasonable to postulate that unicellular organisms should be "self-sufficient" in utilizing copper and may be exposed to considerably higher concentrations of the metal in the environment. Therefore, an "emergency" response is needed such as is offered by transcriptional regulation of the efflux system, e.g., Cop system in bacteria. Interestingly, copper efflux in yeast does not appear to be regulated, since overexpression of the putative copper-efflux ATPase, Pca1, results in cytotoxicity because of, most likely, intracellular copper deficiency (Rad *et al.*, 1994).

Given the relatively slow doubling time of many human cell types and a relatively slow rate of protein synthesis, it is conceivable that the primary function of

MNK is intercellular copper transport. Indeed, primary effects of copper therapy in Menkes patients are the normalization of the neurological system through the delivery of copper to the brain, while the connective tissue disorders appear to be more difficult to correct. This could be due to delivery of copper to lysyl oxidase being fully dependent on comparatively large amounts of catalytically active MNK, while many other cuproenzymes rely on the network of copper chaperones.

In conclusion, MNK plays a pivotal role in copper homeostasis. During evolution it acquired membrane-specific targeting domains and copper-responsive motifs, which provide MNK with its homeostatic properties making it a ubiquitous player in the complex network of copper homeostasis components in humans.

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