The Menkes protein is a transmembrane copper translocating P-type ATPase. Mutations in the Menkes gene that affect the function of the Menkes protein may cause Menkes disease in humans, which is associated with severe systemic copper deficiency. The catalytic mechanism of the Menkes protein, including the formation of transient acylphosphate, is poorly understood. We transfected and overexpressed wild-type and targeted mutant Menkes protein in yeast and investigated its transient acyl phosphorylation. We demonstrated that the Menkes protein is transiently phosphorylated by ATP in a copper-specific and copper-dependent manner and appears to undergo conformational changes in accordance with the classical P-type ATPase model. Our data suggest that the catalytic cycle of the Menkes protein begins with the binding of copper to high affinity binding sites in the transmembrane channel, followed by ATP binding and transient phosphorylation. We propose that putative copper-binding sites at the N-terminal domain of the Menkes protein are important as sensors of low concentrations of copper but are not essential for the overall catalytic activity.

Copper is an essential trace element: its ability to redox cycle between Cu(I) and Cu(II) states is utilized by cuproenzymes participating in redox reactions. However, these same properties make excess copper toxic to biological systems (1). Finely tuned complex mechanisms of copper homeostasis have evolved to allow the regulated uptake of copper, its delivery to target proteins, and detoxification by chelation and/or efflux from the cell (2–4). Copper-translocating P-type ATPases found in a variety of organisms are implicated in the delivery of copper to some cuproenzymes and in the efflux of copper from the cell (2–4).

The Menkes (MNK) protein (ATP7A) is a copper-translocating P-type ATPase expressed in most tissues except the liver (5–8). Mutations in the Menkes gene that cause the loss of function of the MNK protein result in Menkes disease in humans, a potentially lethal X-linked disorder associated with severe systemic copper deficiency. Menkes patients suffer from neurological and connective tissue abnormalities as a result of copper deficiency, which reduces the activity of copper-dependent enzymes (9). Through clinical and laboratory studies on Menkes disease patients, the role of the MNK protein in the absorption of dietary copper from gut epithelium, delivery of copper to cytochrome P450, and efflux from the cell were established (9, 10).

P-type ATPases are multispanning membrane proteins that translocate ions (e.g. H⁺, Na⁺, K⁺, Ca²⁺, Cu⁺, and Cd²⁺) across biological membranes against an electrochemical and concentration gradient using ATP as an energy source (11, 12). The catalytic cycle of P-type ATPases is characterized by the coupled reactions of cation translocation and ATP hydrolysis with a transient aspartyl phosphate formed as a part of the reaction cycle. The phosphorylation results in the enzyme changing its conformation from the high affinity cation and nucleotide binding state, E1, to the low affinity, E2, state. This transition coincides with cation translocation from the cytosolic to the luminal side of the membrane. The release of the cation is followed by the hydrolysis of the aspartyl phosphate bond, and the return of the enzyme to the E1 state. Fig. 1 shows a proposed model for the reaction cycle of MNK based on the model proposed for classical P-type ATPases (13–15). The E1-P conformation is also characterized as ADP-sensitive because the enzyme can be dephosphorylated by ADP (reverse reaction), whereas the E2-P state is ADP-insensitive (13, 14). Investigating the properties of transient aspartyl phosphate provided important structure-function information on the catalytic mechanism of P-type ATPases.

Although H⁺, Na⁺/K⁺ and Ca²⁺ P-type ATPases have been studied extensively (15), copper P-type ATPases have been discovered relatively recently (16), and the mechanism of catalysis is still poorly understood. Thus, even the hallmark of P-type ATPases, the formation of the acylphosphate intermediate, has not been assessed in detail. Apart from eight highly conserved domains, the structure of human copper P-type ATPases differs considerably from other enzymes of that family (17). The most prominent feature of human Menkes protein and the related Wilson (WND) protein, a copper P-type ATPase expressed in the liver, is six repeats of the putative metal-binding motifs (GMXCXXC) at the N-terminal domain (5–8).

Copper binding properties of the putative metal-binding sites (MBSs) of human copper P-type ATPases have been a major focus of studies on the structure of these enzymes. Several reports have established a stoichiometrical binding of Cu(I) to the N terminus of MNK and WND (18, 19). In addition, the copper exchange between this domain and a cytosolic copper chaperone, ATOX1, has been demonstrated in vitro (20, 21). At least some of the MBSs appear to be involved in regulation...
of copper-stimulated trafficking of MNK, which is believed to be essential for copper absorption into the body and copper detoxification (22, 23). However, despite these findings, the role of MBSs in the catalysis of copper translocation, the pivotal function of MNK, is yet to be fully understood.

The observation that functional complementation of the yeast copper P-type ATPase, Ccc2, can be provided by the MNK and WND proteins has become the basis for an indirect assay. Therefore, any MNK or WND mutant unable to complement the Δccc2 yeast cannot grow in copper/iron-depleted medium, as in the absence of the Ccc2 protein, copper cannot be delivered and incorporated into cuproenzyme Fet3, the function of which is essential for high affinity iron uptake. The expression of MNK or WND complements the growth of Δccc2 yeast through, presumably, high affinity copper transport (24).

In the current study, we overexpressed the wild-type and targeted mutant MNK in yeast and provided the first detailed analysis of transient phosphorylation of the human MNK protein. Through these studies, we examined the role of putative MBSs in catalysis, and we propose that their role is high affinity copper sensing/activation of the MNK protein.

EXPERIMENTAL PROCEDURES

Media, Strains, and Plasmids—Yeast Saccharomyces cerevisiae strains YSC1 (MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 Δccc2::URA3) and SUB61 (MATa lys2-801 leu2-3 2-112 ura3-52 his3-Δ200, trp1-1(am), Δccc2::KanR) were provided by Drs. A. Dancis and R. Farrell. These yeast strains have been transformed with ingredients were added, and samples were processed as described above. The amount of purified vesicles transfected cells was normalized against the level of wild-type MNK on the same blot by using laser densitometry (27).

Western Immunoblotting Analysis—Yeast and mammalian cells were lysed in 0.2% SDS prior to Western immunoblotting analysis. Proteins were resolved on a 4–20% SDS-polyacrylamide gradient gel (Novex, San Diego, CA) and transferred onto a nitrocellulose membrane as described previously (28). MNK was detected using polyclonal rabbit antibodies raised against the N-terminal or C-terminal region of MNK (28). MNK was visualized using an enhanced chemiluminescence kit (Roche Diagnostics GmbH). As pure MNK is not currently available the relative amounts of MNK in purified vesicles from transfected cells was normalized against the level of wild-type MNK on the same blot by using laser densitometry (27).

Catalytic Activity of the Menkes Copper P-type ATPase

Copper Transport Assay—Copper transport assays were conducted according to the method described previously (27, 37). 64Cu was obtained from Australian Radioisotopes, ANSTO (Lucas Heights Research Laboratories, Lucas Heights, New South Wales, Australia) and stopped at various time points. The reactions were initiated by adding 1 μM [Cu(I)]-X (in) and ATP (ADP) as specified in Materials and Methods. The concentrations of CuCl2, the copper chelator BCS or inhibitors of the reaction were added. When studying concentration-dependent inhibition of MNK by orthovanadate, vesicles were preincubated with the inhibitor for 10 min on ice (0 °C) in 20 mM MOPS (pH 6.8) buffer supplemented with 150 mM NaCl, 5 mM MgCl2 and 50 μM dithiothreitol or 0.2 mM ascorbic acid. Protein concentration of the vesicle preparation was determined using a protein assay kit (Bio-Rad). The assay was conducted using MNK-enriched membrane vesicles from yeast and carried out on wet ice (0 °C) in 20 mM MOPS (pH 6.8) buffer supplemented with 150 mM NaCl, 5 mM MgCl2 and 50 μM dithiothreitol or 100 μM ascorbate as reducing agents. Various concentrations of CuCl2, the copper chelator BCS or inhibitors of the reaction were added. When studying concentration-dependent inhibition of MNK by orthovanadate, vesicles were preincubated with the inhibitor for 5 min on ice, and then copper and other ingredients were added, and samples were processed as described above. Each incubation contained 20 μg of vesicle protein. The reactions were initiated by adding 1 μM [γ-32P]ATP (1 Ci/mmol; Geneworks, South Australia, Australia) and stopped at various time points. The reaction mixture was subjected to SDS-PAGE and autoradiography using mouse anti-MNK antibodies (28) and Pansorbin (Calbiochem Biosciences, La Jolla, CA) as a source of Protein A. The immunoprecipitate was washed, and protein(s) were eluted and subjected to SDS-PAGE and autoradiography using the Kodak Biomax-MS film and Biomax-MS amplifying screen (Eastman Kodak Co.). The film was exposed for 24–72 h at −70 °C. Autoradiograms were analyzed using laser densitometry.
Catalytic Activity of the Menkes Copper P-type ATPase

**RESULTS**

The wild-type and mutant MNK proteins were expressed stably and at a relatively high level in yeast. A similar content of the wild-type and mutant MNK protein in membrane vesicles (Fig. 2) provided a significant advantage in terms of the use of yeast over the mammalian expression system, in which the expression of MNK mutants used in the present study was unstable and variable despite the constant selection with an antibiotic G418 (22, 28). The MNK protein expressed in yeast had a smaller apparent molecular weight than MNK expressed in CHO cells from the same cDNA construct (Fig. 2). The protein was not truncated, as the Western immunoblotting analysis detected both yeast and CHO cells expressed MNK protein was not truncated, as the Western immunoblotting analysis detected both yeast and CHO cells expressed MNK

**Catalytic Activity of the Menkes Copper P-type ATPase**

**C**

**ccc2**

**Δccc2-EV**

**Δccc2-wtMNK**

**Δccc2-mMBS1-3**

**Δccc2-mMBS1-6**

**ccc2**

**Δccc2**

**Δccc2-wtMNK**

**Δccc2-mHD**

**ccc2**

**Δccc2**

**Δccc2-wtMNK**

**Δccc2-mHD**

**Fig. 3. Δccc2 complementation assay in yeast.** Appropriate serial dilutions of yeast overnight cultures were grown on minimal medium plates supplemented with copper and iron chelators (see under “Experimental Procedures”).

**Fig. 4. In vitro ⁶⁴Cu translocation assay in the presence of 2 μM **

**CHOC-MNK Vₐ = 0.314 nmol Cu/min/mg protein**

**yeast wtMNK Vₒ = 0.352 nmol Cu/min/mg protein**

**yeast wtMNK, 50 μM Va Vₒ = 0.170 nmol Cu/min/mg protein**

**yeast mMBS1–3 Vₒ = 0.138 nmol Cu/min/mg protein**

**yeast EV Vₒ = 0.005 nmol Cu/min/mg protein**

**yeast mHD Vₒ = 0.010 nmol Cu/min/mg protein**

**yeast D1044E Vₒ = 0.016 nmol Cu/min/mg protein**

**Time, min**

**Copper/Cu²⁺ translocation assay in the presence of 2 μM**

**Vₐ**

**S.E.**

**Fig. 2. Western immunoblotting analysis of the Menkes protein expressed in the Saccharomyces cerevisiae yeast and cultured CHO cells.** Vesicles were prepared from CHO cells stably transfected with the MNK cDNA (10 μg) (lane 1), wtMNK yeast with a low level of MNK expression (10 μg) (lane 2), wtMNK yeast with a high level of MNK expression (lane 3), yeast transformed with expression vector only (EV) (5 μg) (lane 4), wtMNK yeast with a high level of MNK expression (5 μg) (lane 5), yeast transformed with the mHD mutant of MNK (5 μg) (lane 6), yeast transformed with the D1044E mutant of MNK (5 μg) (lane 8), wtMNK yeast with a high level of MNK expression (5 μg) (lane 9), and yeast transformed with the mMBS1–6 mutant of MNK (5 μg) (lane 10).
To fully understand the mechanism of copper translocation by the Menkes P-type ATPase, we have investigated transient phosphorylation of MNK and its copper dependence using isolated membrane vesicles. The wtMNK protein was phosphorylated by \( ^{32}P\)ATP on wet ice (0-2 °C) in a time-dependent manner with the maximum phosphorylation occurring within 20 s. (Fig. 5A). A labeling longer than 20 s resulted in irreversible acylphosphate-independent phosphorylation of MNK.2 In the presence of a copper chelator, 1 mM BCS, there was no significant phosphorylation observed, suggesting that copper was essential for the formation of acylphosphate (Figs. 5A and 6). Hydrolysis of the \([^{32}P]\)MNK complex with 100 mM hydroxylamine is consistent with the acylphosphate nature of the intermediate (Fig. 5A). “Pulse-chase” of MNK acylphosphate with 1 mM “cold” ATP demonstrated the transient nature of the phosphorylated intermediate, with almost a complete turnover of MNK being observed by 60 s (Fig. 5, D and E). The formation of MNK acylphosphate was reversible in the presence of 1 mM ADP or 1 mM BCS, as indicated by rapid dephosphorylation of the \([^{32}P]\)wtMNK complex (Fig. 7). The results suggested that, by analogy with other P-type ATPases, under the experimental conditions, at least 70% of the phosphorylated MNK protein was present in the ADP-sensitive E1-like state, as 30% phosphorylated intermediate remained phosphorylated following pulse-chase with ADP (Figs. 1 and 7B). Presumably, the latter represents the E2-P ADP-insensitive state of MNK. As expected for a P-type ATPase, orthovanadate inhibited the phosphorylation of MNK. However, at least 200 μM of the inhibitor prepared from CHO cells stably transfected with the wild-type MNK cDNA, yeast transformed with the wild-type MNK cDNA, and yeast transformed with the mB51–3 mutant of MNK, F, phosphorylation of wtMNK and mB51–6 mutant in the absence of added copper (control) or in the presence of 5 μM copper, cadmium, zinc, or 0.37 μM mercury. 1 mM ATP was added following the 20 s of labeling with \( ^{32}P\)ATP. All reactions were conducted on wet ice. The labeling at 20 s was normalized to 100%.

\[2\]

I. Voskoboinik, J. Mar, D. Strausak, and J. Camakaris, unpublished observations.
was required to inhibit the formation of acylphosphate significantly (Fig. 5A).

The inability of the D1044E mutant to form an acylphosphate intermediate from [γ-32P]ATP (Fig. 5C) indicated that the invariant aspartate residue within the conserved (among all P-type ATPases) DKTG motif is the most likely residue phosphorylated during the reaction cycle. The lack of phosphorylation of the mHD mutant indicated that the alteration of the conserved motif within the ATP-binding loop probably prevented the ATP binding and consequently resulted in the inability of the mutant to be acyl-phosphorylated (data not shown) and transport 64Cu (Fig. 4).

The formation of acylphosphate was copper concentration-dependent, with the maximum level of phosphorylation observed at 5 μM copper (Fig. 6). A further increase in copper concentration resulted in the inhibition of phosphorylation, due, most likely, to substrate inhibition and/or protein denaturation. This is in agreement with the 64Cu translocation vesicle assay, in which the transport of 64Cu could not be measured at >5–6 μM copper (27). Together, these results indicated the heterologously expressed human wtMNK in yeast was a fully active copper pump that had all the features characteristic of a P-type ATPase and was essentially indistinguishable from MNK expressed in mammalian cells. The formation of wtMNK acylphosphate intermediate appeared to be copper-specific, as no detectable phosphorylation was observed in the presence of other heavy metals, such as cadmium, zinc, and mercury (Fig. 5F).

The MNK mutant with the first three N-terminal MBSs mutated (mMBS1–3) remained catalytically active with respect to 64Cu translocation, although its activity was reduced by 40–50% compared with wtMNK (Fig. 4). The apparent kinetics parameters for mMBS1–3 were $K_{m} = 4.0 \pm 0.7 \mu M$ copper and $V_{max} = 0.37 \pm 0.03 \text{nmol of copper/min/mg of protein}$, and with respect to ATP, $K_{m} = 13 \pm 4 \mu M$ ATP (±S.E.), similar to the wtMNK (see above). The mutant protein formed transient aspartyl phosphate from [γ-32P]ATP and turned over in the presence of 1 mM ATP identically to wtMNK (Fig. 5, D and E).

The substitution of Cys to Ser in all six MBSs of MNK resulted in the mutant mMBS1–6, which had no detectable 64Cu-translocating activity under the assay conditions (1–5 μM 64Cu). Because of the inhibitory effect of copper (see above), we were unable to test whether the mMBS1–6 mutant could transport 64Cu at concentrations >5 μM copper. However, the mutant protein was transiently phosphorylated in a copper-specific and copper concentration-dependent manner and was turning over, as judged by the pulse-chase experiment in the presence of 1 mM ATP, in a fashion similar to the wtMNK and mMBS1–3 proteins (Fig. 5, B, D, E, and F). The phosphorylation of mMBS1–6 also appeared to be reversible in the presence of ADP or BCS (Fig. 7).

In order to clarify the role of MBSs in catalysis and to understand potential reasons for apparently conflicting results between phosphorylation assays suggesting that the mMBS1–6 mutant is catalytically active, and the lack of 64Cu-translocating activity and Δccc2 complementation (Fig. 3), we attempted to simulate the conditions of the yeast growth assay in vitro, i.e. severe copper limitation. Thus, the phosphorylation assay was conducted in the presence of copper to stimulate the phosphorylation, but copper was rendered unavailable by increasing concentrations of the copper chelator BCS, which is commonly added to yeast growth medium in order to conduct the Δccc2 assay. The results of this experiment indicated that the phosphorylation of mMBS1–6 was up to 2-fold lower than wtMNK in the presence of BCS (Fig. 8). This was consistent with a faster rate of dephosphorylation of mMBS1–6 than wtMNK in the presence of BCS (Fig. 7A), suggesting that the mutant copper transporter had a lower affinity for copper than the wild-type MNK protein.

Importantly, orthovanadate, a common inhibitor for P-type ATPases (40), appeared to be a more potent inhibitor of the phosphorylation of mMBS1–6 than wtMNK (Fig. 9). Orthovanadate is a structural homologue of orthophosphate that binds to the invariant Asp$^{1044}$ residue of MNK in the ADP-insensitive E2 state (see Fig. 1). Therefore, in the absence of high affinity copper-binding sites in mMBS1–6, a relatively higher proportion of the mutant appears to be present in the E2-like conformation (Fig. 7B), which can be more susceptible to the orthovanadate inhibition, as documented for other P-type ATPases.

The level of Tx-MNK mutant in membrane vesicles was essentially identical to the level of the wtMNK (Fig. 10A). However, the mutant was unable to complement the Δccc2 phenotype in yeast (Fig. 10B) and could not transport 64Cu in the vesicle transport assay (Fig. 10C). These findings agree with our previous report using the Toxic milk mouse mutant form of the Wilson protein (41). Importantly, the Tx-MNK mutant protein could not form transient acylphosphate using [γ-32P]ATP (Fig. 10D). That suggests the Tx-MNK mutation in the putative transmembrane domain 8 of MNK affected high
affinity copper binding, potentially within the cation channel, that prevented the mutant MNK from acquiring the putative high affinity ATP binding conformation.

**DISCUSSION**

This report presents the analysis of acylphosphate formation by the human MNK copper-translocating P-type ATPase and provides evidence for the role of putative MBSs as high affinity copper sensors. This function is predicted to be essential for the physiological role of MNK in the cell, where bioavailable copper is found at very low concentrations (42). The heterologous expression in yeast of the wild-type and mutant MNK circumvented the problems of instability and, often, poor levels of expression of MNK mutants in mammalian cells (28). Importantly, the catalytic properties of the wtMNK protein expressed in yeast were found to be essentially identical to the protein expressed in mammalian cells (28).

**FIG. 8.** Inhibition of phosphorylation of wtMNK and mMBS1–6 by the copper chelator BCS. Labeling with [γ-32P]ATP was conducted for 20 s. The level of phosphorylation in the absence of BCS was normalized to 100%.

**FIG. 9.** The inhibition of phosphorylation of wtMNK and mMBS1–6 by sodium orthovanadate. Membrane vesicles were preincubated with orthovanadate for 5 min on ice. The labeling with [γ-32P]ATP was conducted for 20 s. The level of phosphorylation in the absence of orthovanadate was normalized as 100%.

**FIG. 10.** The effect of M1393V (Tx-MNK) mutation on the activity of MNK. A. Western immunoblot of membrane vesicles prepared from wtMNK (lanes 1 and 2, 2.5 and 5.0 μg of protein, respectively) and Tx-MNK (lane 3, 2.5 μg of protein). B. Δcc2 complementation assay (as described in the legend to Fig. 3 and under “Experimental Procedures”). C. Vesicle 64Cu translocation assay (as described in the legend to Fig. 4). D. The formation of transient acylphosphate from [γ-32P]ATP by wt-MNK and Tx-MNK (see under “Experimental Procedures”). The reactions were conducted on ice. Pulse-chase was started by adding 1 mM ATP after 20 s of phosphorylation with 1 μM [γ-32P]ATP of membrane vesicles (400 μg/ml).
functional only under copper-deficient conditions generated by the addition of BCS. Consequently, the reduced affinity of an MNK mutant for copper may be manifested as the inability to complement the growth of Δccc2 yeast under copper-depleted conditions.

Indeed, when in the present study the mMBS1–6 mutant was analyzed for copper-stimulated phosphorylation, ADP- and BCS-dependent dephosphorylation, and turnover, it appeared to be almost as active as the wtMNK protein (Figs. 5–9). However, the mutant protein could not rescue the Δccc2 phenotype. To clarify the role of MBSs in the catalytic mechanism of MNK, we analyzed the acyl phosphorylation of wtMNK and mMBS1–6 by attempting to simulate in vitro the copper-deficient conditions of the Δccc2 complementation assay (Fig. 8), in which extracellular copper was depleted by the copper chelator BCS. The stronger inhibition of phosphorylation of mMBS1–6 than wtMNK by lower amounts of BCS indicated decreased affinity of the mutant for copper (Fig. 8). Furthermore, a structural homologue of P₉ orthovanadate, indicated a stronger inhibitory effect on the acyl phosphorylation of mMBS1–6 than wtMNK (Fig. 9). Interestingly, the concentration of orthovanadate required to significantly inhibit acyl phosphorylation of wtMNK was substantially higher than in the case of non-heavy metal P-type ATPases. A similar observation has been reported recently for a bacterial copper P-type ATPase CopA (43). These results can be best explained if a high affinity copper binding to MBSs in wtMNK would lead, directly or indirectly, to a higher proportion of the enzyme present in the P-/vanadate-insensitive E1 state. Unlike for other P-type ATPases, we were unable to measure detectable formation of the E2-P intermediate of MNK using 32P (data not shown) that may have resulted from the E1 ↔ E2 equilibrium being shifted toward the E1 conformation, which has a low affinity for P₉ (Fig. 1). This is in agreement with a poor inhibition, compared with other P-type ATPases, of acylphosphate formation by orthovanadate, the structural homologue of P₉ (Fig. 9). Alternatively, the affinity of MNK for P₉ may be much lower than for other P-type ATPases. It appears that the mMBS1–6 mutant has a decreased affinity for copper. The implication of this is that under the conditions of increased copper concentrations, the catalytic cycle of the mutant protein is similar to wtMNK, whereas under physiological (very low bioavailable) copper concentrations, the mutant protein is unable to perform its catalytic function. Overall MBSs appear to be “internal regulators” of MNK activity, as they provide the enzyme with high affinity copper sensors that, upon the binding of copper, increase the proportion of MNK in the E1 state and thus facilitate initiation of catalysis. In the absence of the MBSs, the affinity of the mutant protein for copper would be reduced, and as a result, the protein would appear inactive in the Δccc2 assay conducted under copper-depleted conditions.

An analogous situation has been found in the yeast calcium/manganese P-type ATPase, Pmr1, the N-terminal EF hand-like domain of which contains high affinity calcium binding sites (44). It has been shown that although mutations of these calcium binding sites altered the kinetics of the enzyme by increasing the apparent Kₐ value for Ca²⁺, the overall catalytic activity of the mutant proteins was reduced by less than 50% (44). In our study, we were unable to measure the ⁶⁴Cu-translocating activity of mMBS1–6 in yeast. Unlike calcium, copper binds to proteins nonspecifically with a high affinity that often leads to the inhibition of their catalytic activity. In the case of MNK, the presumed inhibitory effect of copper was observed at >5 μM copper (28) (Fig. 6). Should the mMBS1–6 mutant have an increased Kₐ value for copper, which could be expected based on the phosphorylation studies using BCS and orthovanadate (Figs. 7–9), we would be unable to analyze the catalysis of ⁶⁴Cu transport in membrane vesicles in vitro at >5 μM copper.

In our previous study, we overexpressed the mMBS1–6 mutant in CHO cells and analyzed ⁶⁴Cu transport using whole cells and MNK-enriched membrane vesicles (28). We had found, in contrast to the current study, that the mutant MNK had a reduced but measurable ⁶⁴Cu-transporting activity in vitro (28). These data could be explained if some copper ligands and/or cell type-specific protein-protein interactions contributed to ⁶⁴Cu translocation by the mutant MNK expressed in CHO cells as opposed to yeast cells. Importantly, the expression level of endogenous hamster MNK, which shares >95% identity with human MNK, was not increased in these CHO cells transfected with the mMBS1–6 mutant as determined by Northern analysis (results not shown). It has been reported earlier that the mMBS1–6 mutant expressed in CHO cells, unlike its wild-type counterpart, could not undergo copper-stimulated trafficking from the trans-Golgi network to the plasma membrane, where it is expected to efflux copper from the cell (22, 28). The mutant-transfected cells also had a copper hyperaccumulation phenotype and reduced copper resistance, but only when they had been exposed to increased concentrations of copper (28). In light of the findings presented in the current study, one can propose that due to decreased affinity for copper, the mMBS1–6 mutant was unable to transport low physiological concentrations of copper, but it “became” catalytically active when higher concentrations of copper were presented to cells. Under physiological conditions, copper may be delivered to the MBSs of MNK via the high affinity copper chaperone ATOX1 (20, 21), which would permit the initiation of copper translocation under these conditions.

Importantly, the mutation of conserved Met-1393 to Val, as occurs for the Toxic milk mouse mutation in the Wilson protein (30), has also resulted in an inactive MNK protein (41) (Fig. 10). The Met-1393 residue is highly conserved in copper P-type ATPases and is proposed to be located within the putative transmembrane domain 8. A soft Lewis base, methionine, in a transmembrane domain may be involved in the co-ordination of copper and therefore constitute a part of a high affinity copper binding site in the cation channel of the MNK protein. Until now, there has been no information on the order of events in the catalytic cycle of copper P-type ATPases. According to Fig. 1, which is based on the Ca²⁺-P-type ATPase paradigm, copper is expected to bind to the MNK protein and lead to conformational changes essential for high affinity ATP binding and hydrolysis. It can be expected, therefore, that the disruption of high affinity copper binding sites in the cation channel would prevent ATP binding and phosphorylation. Here, we demonstrated that the M1393V mutation not only causes the loss of ⁶⁴Cu-translocating activity but results in the mutant protein being unable to become transiently phosphorylated. Although more evidence may be required to prove unequivocally that Met-1393 constitutes a part of the transmembrane copper channel, information provided here suggests that the binding of copper in the putative copper-binding sites within transmembrane domains is required for ATP hydrolysis. This finding also emphasizes the fact that although the mutation of the N-terminal MBS1–6 has some effect on catalysis, it does not appear to prevent copper binding to those sites, presumably in transmembrane domain(s), which are associated with conformational changes essential for high affinity ATP binding and the acylphosphate formation. Furthermore, these sites appear to be copper-specific, as no stimulation of acyl phosphorylation of MNK by the heavy metals cadmium, zinc, and mercury has been observed (Fig. 5F).
Studies on transient phosphorylation of hamster MNK by [γ-32P]ATP have been previously reported (45), but the conditions of the assay favored significant non-acylphosphate phosphorylation,2 37 °C for 90 s, as opposed to the more conventional conditions of the assay favored significant non-acylphosphate phosphorylation to the copper-binding sites in the channel.

In conclusion, in this study, we analyzed the mechanism of MNK phosphorylation and provided evidence that although the putative MBs do not participate directly in the catalytic cycle of the protein, they appear to be essential for the sensing of very low concentrations of copper in the environment or, alternatively, in capturing low concentrations of copper and supplying it to the copper-binding sites in the channel.

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