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ATP-dependent copper transport by the Menkes protein in membrane vesicles isolated from cultured Chinese hamster ovary cells

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Abstract The Menkes (MNK) protein is a vital component of copper homeostasis in mammalian cells. In this paper we provide the first biochemical evidence that the MNK protein functions as a copper-translocating P-type ATPase in mammalian cells. The enzyme activity in membrane vesicles prepared from Chinese hamster ovary cells overexpressing MNK was ATP-dependent, correlated with the amount of MNK and followed Michaelis-Menten kinetics with respect to copper. The copper transport was observed only under reducing conditions suggesting MNK transports Cu(I). This study opens the way to detailed structure-function studies and assessment of functional MNK derived from patients with Menkes disease.

Key words: Menkes disease; Copper; P-type ATPase; Copper transport

1. Introduction

Copper is an essential trace element which is required for the activity of a number of essential enzymes, e.g. Cu-Zn superoxide dismutase, lysyl amine oxidase, cytochrome *c* oxidase and dopamine β-hydroxylase [1]. Nevertheless high concentrations of copper can pose a danger, because intracellular redox potential, controlled primarily by thiols and anti-oxidant enzymes, can cause the reduction of Cu(II) to Cu(I) with reactive oxygen species being formed as side-products [2]. It is probable that all cells have an efficient system for regulating copper homeostasis, which involves uptake, delivery to appropriate cell compartments and enzymes and, finally, efflux from the cell.

Menkes disease is an X-linked genetic disorder of copper transport in humans caused by mutations in the Menkes gene (*ATP7A*), which encodes the Menkes protein, hMNK. The disease is caused by gross systemic Cu deficiency, mainly due to defective intestinal absorption of Cu associated with accumulation of copper in gut mucosa [3–5]. Neurological symptoms observed in Menkes patients are believed to be due to deficiencies in the activity of copper-dependent cytochrome *c* oxidase, Cu-Zn superoxide dismutase and dopamine β-hydroxylase [3,4], while lysyl oxidase deficiency is responsible for the abnormalities of connective tissue [3,4,6]. The accumulation of copper in cell lines derived from Menkes patients compared to normal cells [7–11] concomitant with decreased activity of copper-containing lysyl oxidase [6,12,13] suggests that the delivery of copper to various cellular compartments is a significant aspect of Menkes disease. Taken together, all these findings support the concept that the primary cause of Menkes disease is impaired copper homeostasis.

Consistent with the above findings, a candidate gene for the hMNK protein has been cloned [14–16], and the deduced amino acid sequence suggested the protein contains several regions which are highly conserved among all P-type ATPases [17]. These enzymes are commonly involved in the transport of various cations (e.g. Ca, Cd and Hg) across biological membranes [18,19]. Moreover, structural hMNK homologues, which have been characterised in yeasts, CCC2 [20], and prokaryotes, CopB [21] are involved in Cu transport. MNK mRNA has been found in most tissues, except the liver, in mammals, including humans. Previous studies from our laboratory provided clear evidence of the ability of copper-resistant chinese hamster ovary (CHO) cell lines, CUR2 and CUR3, derived from the parental strain, CHO-K1, to withstand high concentrations of extracellular copper due to enhanced copper efflux. This correlated well with overexpression of chMNK in these cells resulting from gene amplification [22]. The chMNK protein has more than 90% homology to hMNK [22], so it is assumed that these proteins will have similar Cu-transporting activities.

In the present study, an in vitro vesicle assay was used to provide the first direct biochemical evidence that the mammalian MNK homologue is a copper-transporting P-type ATPase. To establish unequivocally that the ATP-dependent translocation of copper was due to the chMNK protein, use was made of parental CHO-K1 cells and their copper-resistant variants, which overexpress the MNK protein by up to 70-fold [22].

2. Materials and methods

2.1. Plasma membrane enriched vesicle preparation

CHO-K1, CUR2 and CUR3 (copper-resistant cells which overexpress chMNK [22]) were cultured in basal Eagle's medium with Earle's salts supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 0.2 mM proline, 1.2 mM $NaHCO_3$ and 20 mM HEPES [22]. The cells were grown as a monolayer on 900 cm^2 roller bottles until confluent (approximately 10^7 cells). The cells were incubated in growth medium supplemented with 189 μM $CuCl_2$ for 2 h prior to being harvested by scraping into cold phosphate buffered saline, and pelleted at $1000\times g$ for 10 min. The resulting pellet was snap frozen and stored at −70°C until further use.

Vesicles used in this experiment were prepared according to the procedure for preparing plasma membrane enriched vesicles described by Schlemmer and Sirotnak [23] except that the $9800\times g$ centrifuga-

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Abbreviations: AMP-PCP, adenosine-5′-[β,γ-methylene]triphosphate; CHO, Chinese hamster ovary cell line; CUR, copper resistant CHO cell line; DTT, DL-dithiothreitol; MNK, Menkes protein; hMNK, human Menkes protein; chMNK, CHO Menkes homologue; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid

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tion and dialysis steps were omitted. All buffers contained antiproteases: phenylmethylsulphonyl fluoride (100 μg/ml), leupeptin (0.5 μg/ml), pepstatin (1 μg/ml) and aprotinin (1 μg/ml). Protein concentration of the vesicle preparation was determined using Bio-Rad protein reagent (Bio-Rad Laboratories) [24]. Vesicles were stored at −70°C; under these conditions the rate of copper transport was not affected after at least 2 months of storage.

2.2. Immunoblotting analysis

Vesicles lysed in 0.2% SDS, and whole cell extract prepared as described in [22], were size-fractionated on a 4–20% SDS-polyacrylamide gradient gel (Novex, San Diego, CA, USA). The gel was transferred overnight by electroblotting to nitrocellulose membrane. Primary antibodies used in this study were raised in rabbits. Immunodetection of the Menkes protein was carried out using the polyclonal antibody raised to the amino-terminus of MNK [22], and the detection of Na/K-ATPase was carried out using polyclonal antisera against Na/K-ATPase provided by Dr Judy Callaghan, Department of Pathology and Immunology, Monash University, Victoria, Australia [25]. The proteins were visualised with an enhanced chemiluminescence kit according to the manufacturer's instructions (Boehringer, Mannheim, Germany).

2.3. Copper transport measurements

The copper transport assay was conducted in 40 mM histidine buffer (pH 6.8) containing 100 mM KCl and 5 mM $MgCl_2$ [26], and supplemented with 25 μM DTT in the presence of 50 μg/ml vesicle protein and with or without 5 mM ATP at 37°C. Copper was added as a mixture of $CuCl_2$ and $^{64}CuCl_2$, specific activity 260–5800 mCi/mg (Australian Radioisotopes, Lucas Heights, NSW, Australia). The transport assay was started by adding appropriate amounts of vesicles to the reaction mixture. The rates of Cu uptake were estimated by filtering aliquots of the reaction mixture (at various time points) through 0.45 μm nitrocellulose filters (Sartorius AG, Göttingen, Germany). The filters were washed with 1.5 ml of reaction buffer containing 1 mM $CuCl_2$, and their radioactivity was measured using an LKB-Wallac 1282 Compugamma Universal Gamma Counter (Finland). There was no significant difference between the amount of protein loaded onto the filters and recovered from them (unpublished data). The filters were pre-blocked with 1 mM $CuCl_2$ for at least 1 h prior to experiments to minimise non-specific Cu binding.

ATP-dependent copper transport was estimated by subtracting the rate of Cu uptake in the absence of ATP from total rate measured in the presence of ATP during the linear phase of the reaction (2–12 min). There were similar amounts of copper taken up by the vesicles in the presence and absence of ATP within first 2 min of the reaction. Within the same time frame there was no substantial difference between the rates of Cu uptake into the CHO-K1 and CUR3 vesicles. Therefore we considered that this rapid initial phase of the reaction was due to a non-specific Cu binding. Rates of Cu uptake were related to total protein of vesicle fraction used (in the absence of pure MNK as a standard it is not possible to quote rates related to actual MNK protein concentration).

3. Results

In this study we used plasma membrane enriched vesicles prepared from parental CHO-K1 cells and their copper resistant variants, CUR2 and CUR3 [22] in order to investigate and characterise Cu-translocating activity of the MNK P-type ATPase. Western blot analysis supported previous findings from this laboratory that CUR3 cells contained significantly elevated levels of MNK (Fig. 1A) [22]. There was a large enrichment of MNK (~180 kDa) relative to total protein in the vesicle fraction as compared to the whole cell lysate (Fig. 1A). Similarly there was a strong enrichment of vesicles with Na/K-ATPase (~100 kDa) (Fig. 1B), a marker protein for the plasma membrane.

Fig. 2 shows that while the rate of Cu uptake into CUR3 vesicles was markedly stimulated by ATP (Fig. 2A), its effect on the copper transport into CHO-K1 vesicles was insignificant (Fig. 2B). Consequently, the rate of Cu uptake in CHO-K1 vesicles was essentially undetectable using the current conditions, while vesicles from CUR2 cells had 30% of CUR3 activity (Fig. 3, inset). This is consistent with the amounts of MNK in CHO-K1, CUR2 and CUR3 vesicles (Fig. 1A). Both ADP and a non-hydrolysable ATP homologue, AMP-PCP (adenosine-5′-[β,γ-methylene]triphosphate), failed to support copper uptake (Fig. 3), suggesting the hydrolysis of ATP is required for the copper transport activity. Having established that Cu uptake correlates with the presence and quantity of MNK, all other studies on catalytic properties of chMNK were conducted using CUR3 vesicles.

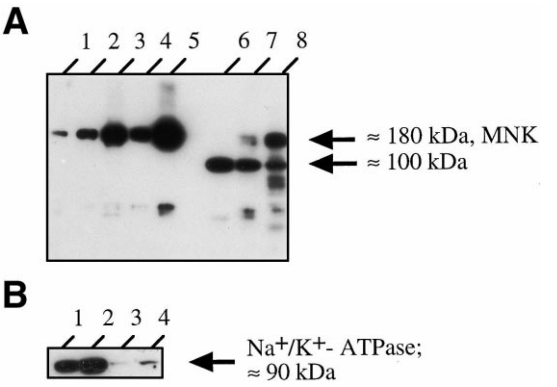

Fig. 1. Immunodetection of the ~180 kDa MNK protein (A) and ~100 kDa Na^+/K^+-ATPase (B) on Western blots (see Section 2). A: Lane 1: vesicles from CHO-K1 cells, 10 μg protein; lanes 2 and 3: vesicles from CUR2 cells, 1 and 10 μg protein; lanes 4 and 5: vesicles from CUR3 cells, 0.1 and 1 μg protein; lane 6: CHO-K1 whole cell homogenate, 100 μg protein; lane 7: CUR2 whole cell homogenate, 50 μg protein; lane 8: CUR3 whole cell homogenate, 25 μg protein. A non-specific 100 kDa band was detected in whole cell homogenates by the polyclonal antiserum used. B: Lane 1: vesicles from CHO-K1 cells; lane 2: vesicles from CUR3 cells; lane 3: CHO-K1 whole cell homogenate; lane 4: CUR3 whole cell homogenate. Each lane was loaded with 10 μg protein.

It should be noted that ATP-dependent copper transport into membrane vesicles can occur only when the ATP binding site is oriented towards the cytosolic side (or buffer system in vitro) which is the predicted topology of MNK in the cell [14–17]. Reproducible Cu-transporting activities observed suggest reproducible sideness of vesicles produced by the method as shown previously [23]. While the ATP-independent copper transport was insensitive to changing osmotic conditions (data not shown), the level of ATP-driven copper uptake in CUR3 vesicles in buffer supplemented with 1 M sucrose was 63 ± 8% (S.D.) of control (no added sucrose) levels. Also there was no ATP-dependent transport observed when vesicles were lysed with 0.1% Triton X-100 (data not shown), and copper uptake was temperature dependent with the rate being reduced by 50% at 22°C compared to the rate at 37°C. Taken together, these results provide strong evidence that in the presence of ATP the copper was actively translocated into the vesicles rather than bound to their surface.

DTT was an essential component of the reaction mixture, as it presumably reduced Cu(II) to Cu(I). Thus in the presence of trace amounts (< 1 μM) of DTT (from vesicle storage buffer) the rate of ATP-dependent copper uptake was 17 ± 5% (S.D.) of control level. Because of the low stability of MNK we were unable to obtain DTT-free samples of vesicles, but it is likely that in the absence of DTT there would

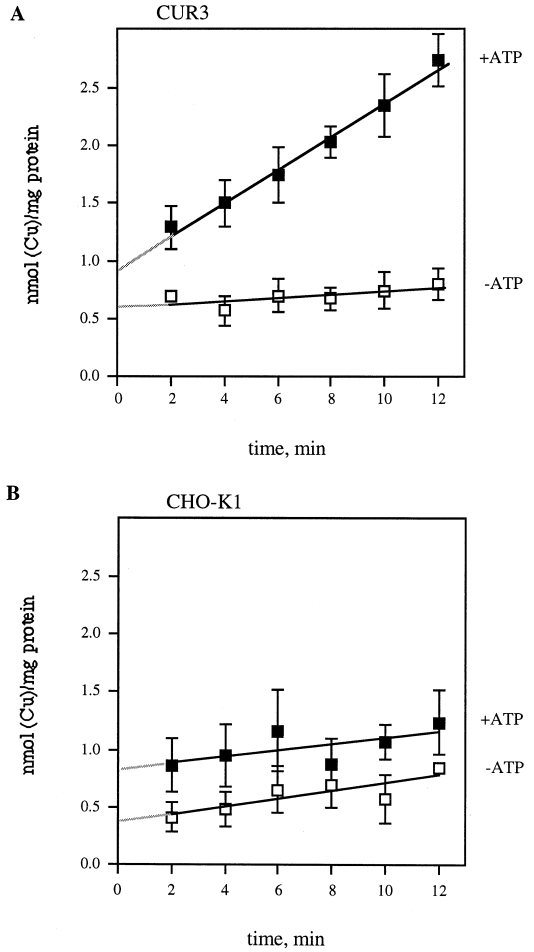

Fig. 2. Time-dependent copper uptake by the vesicles derived from MNK overexpressing CUR3 cells ($n=4$) (A), and their parental CHO-K1 cells ($n=3$) (B). The reaction was conducted in the presence of 2 μM $CuCl_2$ containing $^{64}CuCl_2$, 5 mM ATP, 25 μM DTT and 50 μg/ml vesicles. The data are presented as mean ± S.E.M. (using independent vesicle preparations).

be no active transport observed. These data are consistent with electron spin resonance analysis, which showed a complete disappearance of the Cu(II) profile, when DTT was added at equimolar concentrations to $CuCl_2$ (data not shown). Overall the data suggest that Cu(I) rather than Cu(II) is actively transported by chMNK.

The chMNK protein of CUR3 vesicles displayed Michaelis-Menten kinetics with respect to copper in the presence of ATP and DTT. The apparent K_m (7 ± 3 μM (S.D.)) and the apparent V_{max} (0.67 ± 0.38 nmol/mg/min (S.D.)) were estimated from a Hanes-Woolf reciprocal plot (Fig. 4).

The uptake of copper into vesicles was significantly inhibited by low concentrations of orthovanadate, which is a specific inhibitor for P-type ATPases. The IC_{50} value was estimated as 25 μM (Fig. 5). Structurally diverse alkylators of protein sulphydryl groups *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzoate (PCMB) were potent inhibitors of MNK activity (Fig. 5). Thus 5 μM NEM and 25 μM PCMB reduced the rate of Cu uptake by at least 50% (Fig. 5). These results were obtained from experiments where NEM and PCMB were preincubated with vesicles in a buffer containing approximately 5–8 μM DTT for 5 min, and then diluted with 8 volumes of the reaction buffer to study Cu uptake. Therefore the final concentrations of the inhibitors were not sufficient to deplete DTT in the reaction buffer. The inhibition was irreversible, as the addition of 0.6 μM NEM and 3 μM PCMB (1/8 of estimated IC_{50}) directly to the reaction mixture failed to inhibit copper transport (results are not shown). Ouabain (200 μM) an inhibitor of Na^+/K^+-ATPase, had no effect on copper uptake into CUR3 vesicles (100 ± 15% (S.D.), compared to a no treatment control), indicating that Cu transport was independent of Na^+/K^+-ATPase.

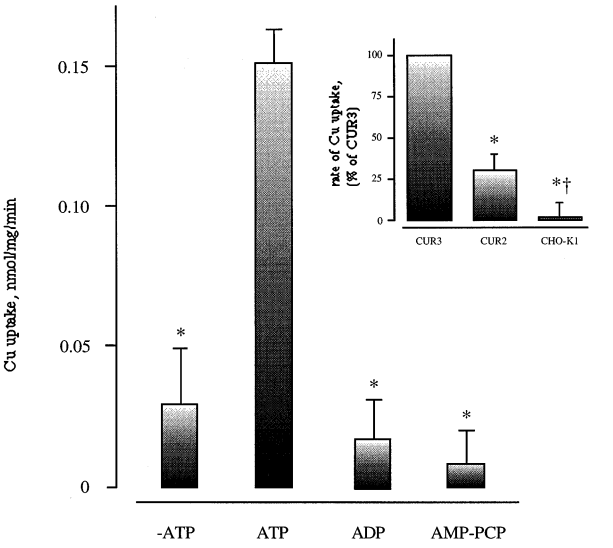

Fig. 3. Copper uptake by the vesicles from CUR3 cells in the presence or absence of 5 mM ATP, or 5 mM ADP and 5 mM AMP-PCP. *Significantly different from 'ATP' ($P<0.05$) Inset: comparative rates of ATP-dependent copper uptake by the vesicles from CHO-K1, CUR2 and CUR3 cells. *Significantly different from CUR3 ($P<0.05$); †significantly different from CUR2 ($P<0.05$). The results are presented as mean ± S.D.

4. Discussion

In this study we provide the first biochemical evidence that

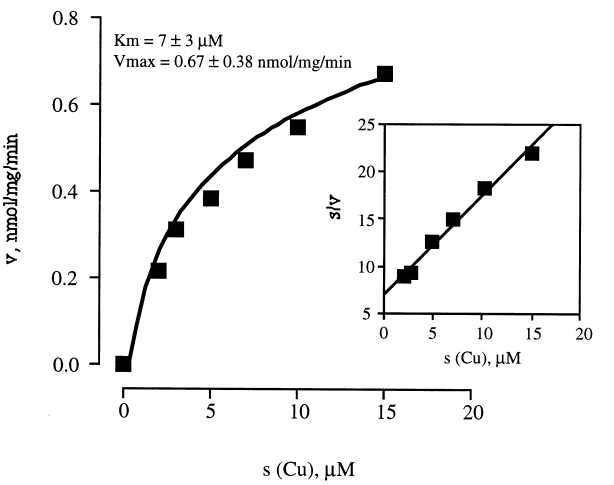

Fig. 4. Michaelis-Menten plot of copper uptake by the vesicles from CUR3 cells. Inset is Hanes-Woolf reciprocal plot. For technical reasons, we were unable to measure the reaction rate at the concentrations of copper higher than 15 μM. The values for apparent K_m and V_{max} are presented as mean ± S.D., and the figures are based on the data from a representative experiment.

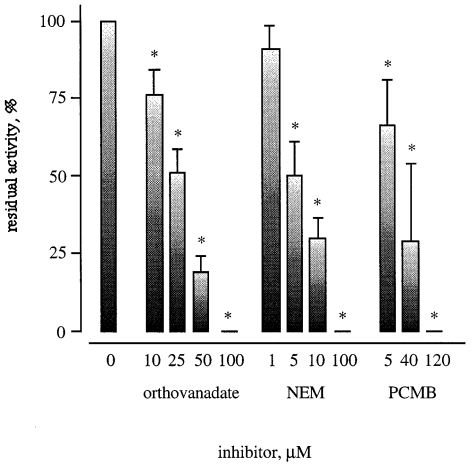

Fig. 5. Inhibition of copper uptake by sodium orthovanadate, *N*-ethylmaleimide and *p*-chloromercuribenzoic acid. $*P < 0.05$ compared to a '0 inhibitor' control (Student's *t*-test). The results presented are mean ± S.D. 'Residual activity' is the percentage of '0 inhibitor' control (100%).

the MNK P-type ATPase functions as an ATP-dependent Cu-translocating pump in mammalian cells. The rates of copper transport correlated well with the levels of chMNK in the vesicles prepared from CHO cells with differing levels of the MNK protein ([22]; Figs. 1A and 3). In addition, the data showing the enzyme activity was stimulated by ATP, but not by ADP or AMP-PCP and inhibited by low concentrations of orthovanadate, a diagnostic inhibitor of P-type ATPases [27], supported predictions based on DNA sequence that the enzyme is a P-type ATPase [14,15,17]. Copper-transporting activity of vesicles derived from CHO cells carrying a stable construct of hMNK has been demonstrated recently in our laboratory and the apparent values for K_m and V_{max} were similar to those presented here (Petris et al., manuscript in preparation). Earlier studies from our laboratory indicated the rate of copper efflux from whole CUR3 cells was 0.2–0.3 nmol/mg/min ([22]; unpublished observations) that is only 2–3 times lower than the apparent value for V_{max} found here (0.67 nmol/mg/min). Solioz and Camakaris [28] recently reported that the MNK protein from CUR3 cells catalyses the formation of an acylphosphate intermediate when incubated with ATP in vitro, which provided additional support that MNK functions as a P-type ATPase.

Dijkstra et al. [29] and Bingham et al. [30] observed an ATP-stimulated Cu uptake in membrane vesicles from rat hepatocytes. The apparent K_m values reported by these authors are similar to those presented in this paper, 8.6 μM Cu [29] and 2.5 μM Cu [30] versus 7 μM Cu. It has been reported that MNK is not expressed in the liver of mammals [31], and, although the liver enzyme has not been identified by Dijkstra and Bingham [29,30], it is likely the activity was related to that of the Wilson protein, a Cu-transporting ATPase found in the liver, which has 54% homology with hMNK [32,33]. Our data strongly supports the notion that we were measuring the MNK P-type ATPase, as Cu-transporting activity was proportional to MNK concentration. Recently Payne and Gitlin [34] have shown the hMNK protein functions in yeast to complement CCC2 deletion mutants, and allows incorporation of Cu into the yeast protein Fet3p in yeast Golgi. Although the yeast assay system supports the role of MNK as a copper pump it does not permit detailed kinetic analysis.

It is not clear whether it is ionic copper or a copper complex which is presented to the MNK protein and transported across the ion channel. It has been suggested that the cytosolic copper chaperone, Atx1, may deliver copper to CCC2 [35]. A homologue of Atx1, HAH1, has been identified in mammalian cells and may function to transport Cu to the Menkes and/or Wilson Cu-translocating ATPases [36]. It is possible that in the in vitro system described in the present studies, DTT functions as a surrogate copper donor for MNK. The process may also require binding of Cu to the Cys-X-X-Cys 'metal binding motif(s)' in the N-terminal domain of MNK. Site-directed mutagenesis studies are in progress, and will provide more details on the mechanism of Cu binding by MNK and in particular which domains, motifs and residues are important in Cu translocation.

Two structurally diverse alkylating agents, NEM and PCMB, appeared to be potent irreversible inhibitors of Cu uptake activity, indicating, not entirely surprisingly, the involvement of the protein thiols in the transport of copper. Qian et al. [7] demonstrated PCMB affected the accumulation and efflux of copper in human fibroblasts in a manner suggesting the inhibition of MNK Cu-transporting activity. It is possible that cysteine thiols of the six -GMXCXXC- 'metal binding' motif(s) found in the N-terminal domain of MNK and/or the -CPC- motif in the ion transduction channel are particularly important for the energy-dependent Cu transport. These motif(s) may also be susceptible to reactive oxygen species, whose formation is induced under various pathological conditions, e.g. inflammation and carcinogenesis [37]. Thus it can be speculated that the accumulation of copper, which is redox active in its own right, in inflamed tissues may be caused by the inhibitory effects of reactive oxygen species on the catalytic activity of MNK [38]. The link between copper accumulation, hepatic inflammation and hepatocarcinogenesis has been supported using Long-Evans Cinnamon rats, an animal model of Wilson's disease [39].

An important question of Cu metabolism that is yet to be clarified is the redox status of copper transported by the Cu P-type ATPases. Under non-pathological conditions, high intracellular concentrations of antioxidants would probably ensure the reduction of Cu(II) to Cu(I). It has been postulated that hMNK and its homologues in other species bind and transport Cu(I) [40,41]. Moreover, Solioz and Odermatt [21] observed ATP-dependent Cu transport catalysed by CopB (bacterial MNK homologue) under reducing conditions only. The data presented in this paper supports the view that MNK transports Cu(I) rather than Cu(II), but further studies are required to establish this conclusively.

Recent work carried out in our laboratory demonstrated that Cu can regulate MNK by causing alteration of its subcellular location [42]; MNK is relocalised via vesicular trafficking from the trans-Golgi network to the plasma membrane in the presence of high extracellular concentrations of copper. Copper-transporting activity of MNK explains, at least partially, the physiological significance of this phenomenon. MNK in the trans-Golgi network is predicted to donate Cu to Cu-dependent enzymes in the secretory pathway, e.g. lysyl amine oxidase. It is hypothesised that excess Cu is sensed by MNK in the trans-Golgi network, and then may stimulate budding and trafficking of vesicles to the plasma membrane. It is pos-

sible that MNK pumps copper into these vesicles and releases it from the cell once the vesicles fuse with the plasma membrane. Alternatively, the vesicles fuse with the plasma membrane and then start pumping copper actively from the cell. In any case, the combination of MNK vesicular trafficking with its Cu-transporting activity forms an efficient detoxification and Cu homeostatic mechanism [22]. In addition, MNK copper-translocating activity across the basolateral membrane of polar gut epithelial cells may provide the mechanism for Cu absorption, as Menkes patients accumulate Cu in these cells [3].

In conclusion, the information presented here provides an insight into the critical role of the ubiquitous MNK in copper homeostasis in mammalian cells and provides the first direct biochemical evidence that it functions as a copper-transporting P-type ATPase. The study opens the opportunity to gain more information on the role of various domains and motifs of the enzyme in its catalytic function through use of engineered mutant MNK constructs expressed in cultured cells. In addition these findings have important therapeutic implications. Kaler et al. [43,44] have suggested that favourable outcomes can be obtained by copper therapy of Menkes disease patients who express MNK, which has residual function, whilst copper therapy may be of no value in patients who do not express a functional MNK transporter. Assays of copper-transporting activity using vesicles from cells overexpressing MNK constructs containing patient's mutations should allow assessment of candidate patients for copper therapy.

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