Purification and membrane reconstitution of catalytically active Menkes copper-transporting P-type ATPase (MNK; ATP7A)

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The MNK (Menkes disease protein; ATP7A) is a major copper-transporting P-type ATPase involved in the delivery of copper to cuproenzymes in the secretory pathway and the efflux of excess copper from extrahepatic tissues. Mutations in the *MNK* (*ATP7A*) gene result in Menkes disease, a fatal neurodegenerative copper deficiency disorder. Currently, detailed biochemical and biophysical analyses of MNK to better understand its mechanisms of copper transport are not possible due to the lack of purified MNK in an active form. To address this issue, we expressed human MNK with an N-terminal Glu-Glu tag in Sf9 [*Spodoptera frugiperda* (fall armyworm) 9] insect cells and purified it by antibody affinity chromatography followed by size-exclusion chromatography in the presence of the non-ionic detergent DDM (*n*-dodecyl β -D-maltopyranoside). Formation of the classical vanadate-sensitive phosphoenzyme by purified MNK was activ-

ated by Cu(I) [$EC_{50} = 0.7 \mu\text{M}$; h (Hill coefficient) was 4.6]. Furthermore, we report the first measurement of Cu(I)-dependent ATPase activity of MNK ($K_{0.5} = 0.6 \mu\text{M}$; $h = 5.0$). The purified MNK demonstrated active ATP-dependent vectorial ⁶⁴Cu transport when reconstituted into soya-bean asolectin liposomes. Together, these data demonstrated that Cu(I) interacts with MNK in a co-operative manner and with high affinity in the sub-micromolar range. The present study provides the first biochemical characterization of a purified full-length mammalian copper-transporting P-type ATPase associated with a human disease.

Key words: copper homeostasis, *n*-dodecyl β -D-maltopyranoside (DDM), Menkes-disease protein (MNK; ATP7A), P-type ATPase, Sf9 [*Spodoptera frugiperda* (fall armyworm) 9] cell.

INTRODUCTION

The MNK (Menkes-disease protein; ATP7A) is a major copper-transporting P-type ATPase with a pivotal role in copper homeostasis. Mutations in the *MNK* (*ATP7A*) gene [1–3] that encodes MNK result in the fatal neurodegenerative disorder, Menkes disease, which is pathologically characterized by systemic copper deficiency with neurological, connective tissue, hair and pigmentation abnormalities [4,5]. Previous studies have identified MNK localization to the *trans*-Golgi network, where it delivers copper to cuproenzymes in the secretory pathway, and undergoes copper-regulated trafficking to the basolateral membrane of intestinal mucosal cells, where it mediates systemic absorption of copper [6,7]. The importance of MNK in the copper homeostasis of the CNS (central nervous system) is highlighted by an association between hippocampal neuronal activation and MNK-mediated copper efflux, suggesting a role for MNK in memory and learning [8]. Emerging evidence also links MNK to the mechanism of resistance to cisplatin, a drug widely used in chemotherapy for cancer treatment [9–11].

P-type ATPases are a superfamily of membrane proteins that utilize the chemical energy of ATP hydrolysis to facilitate active transport of cations across diverse cellular membranes [12]. During the catalytic cycle, transfer of the γ -phosphate from ATP to the conserved aspartic acid residue in the DKTG motif results in the formation of a transient vanadate-sensitive acyl-phosphate

intermediate. Other key signature motifs conserved in P-type ATPases include those for ATP-binding and dephosphorylation.

MNK is classified in the P1_B-subfamily of heavy metal transporting P-type ATPases [13]. In addition to common P-type ATPase motifs, heavy metal transporting P-type ATPases are distinguished by the presence of putative N-terminal MBS (metal binding sites), and the CPX motif in the sixth transmembrane domain [14]. From the amino acid sequence, MNK is predicted to have six N-terminal MBSs followed by eight transmembrane domains (Figure 1A). Members of the P1_B-subfamily closely related to MNK include the WND (Wilson disease protein; ATP7B), copper transporter Ccc2 and putative copper-transporting ATPase PCA1 from *Saccharomyces cerevisiae*, P-type ATPases CopA and CopB from *Enterococcus hirae*, and CopA from *Escherichia coli*.

Crystal structures of the classical SERCA1a (sarcolemmal/endoplasmic-reticulum Ca²⁺-ATPase 1a) P-type ATPase [15–19] provided the framework for understanding the general molecular mechanism of P-type ATPases, but our current knowledge of heavy metal transport mechanisms is limited. Previous biochemical characterizations of MNK using membrane vesicles isolated from mammalian and yeast cells demonstrated the formation of the characteristic acyl-phosphate intermediate [20,21] and ATP-dependent vectorial transport of Cu(I) [20,22,23]. However, measurement of MNK ATPase activity has not been possible due to high contaminating background ATPase activity in these membrane vesicle preparations. Furthermore, a better understanding

Abbreviations used: Atox1, ATX1 antioxidant protein 1 homologue (yeast); BCS, bathocuproinedisulfonic acid; CHO-K1 cell, Chinese-hamster ovary K1 cell; CMC, critical micelle concentration; DDM, *n*-dodecyl β -D-maltopyranoside; DTT, dithiothreitol; EE tag, Glu-Glu epitope tag; EE-MNK, EE-tagged MNK; HRP, horseradish peroxidase; LUV, large unilamellar vesicles; MBS, metal binding site; 2ME, 2-mercaptoethanol; MNK, Menkes disease protein; MOI, multiplicity of infection; NEM, *N*-ethylmaleimide; OG, *n*-octyl β -D-glucopyranoside; pfu, plaque-forming units; SERCA1a, sarcolemmal/endoplasmic-reticulum Ca²⁺-ATPase 1a; TX100, Triton X-100; WND, Wilson disease protein.

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Figure 1 (A) Topological model of the human MNK protein and (B) the catalytic cycle of MNK based on that of the classical Ca²⁺-ATPase

(A) The N-terminal domain of MNK in the cytoplasmic side of the membrane contains six putative MBSs. (B) E₁ denotes MNK in the high-affinity conformational state; E₂, MNK in the low-affinity conformational state; E-P, the MNK acyl-phosphate intermediate; *n*, unknown number of Cu(I) ions.

of MNK functional properties through detailed biochemical and biophysical studies is important for development of improved diagnostic and therapeutic approaches for Menkes disease, and for understanding the role of MNK in neurodegenerative diseases and cisplatin resistance. At present, these studies are limited by the lack of purified MNK protein in an active form, and to the best of our knowledge, direct catalytic analysis of a purified full-length mammalian P1_B-ATPase has not been reported.

In the present study, we report the first biochemical characterization of a purified and membrane reconstituted full-length mammalian P1_B-ATPase. Here, we describe high levels of MNK expression in Sf9 [*Spodoptera frugiperda* (fall armyworm) 9] cells, its purification by antibody affinity chromatography followed by size-exclusion chromatography, and membrane reconstitution of active enzyme. We also demonstrated catalytic activity of purified MNK at three key steps of its proposed catalytic cycle (Figure 1B): (i) formation of the classical P-type ATPase acyl-phosphate intermediate; (ii) the first demonstration of MNK ATPase activity; and (iii) vectorial Cu(I) translocating activity of membrane reconstituted enzyme.

EXPERIMENTAL

Construction of recombinant baculovirus

The inherent instability of MNK cDNA when propagated in *E. coli* cells requires the use of low-copy number plasmids [24]. The baculovirus transfer vector, pBacPAK9 (Clontech, Mountain View, CA, U.S.A.), was modified to replicate at low-copy numbers in *E. coli* by exchanging its high-copy number origin of replication for that of low-copy number from pSWK29 [25]. The pBacPAK9 plasmid was amplified by PCR to exclude the origin of replication, using oligonucleotides 5'-GCTGGCCTT-TTGATCACATGTTTC-3' and 5'-GGGTCTGATCATCAGTGG-AACG-3'. The oligonucleotides have engineered BclI sites to enable subcloning of the BglII fragment containing the origin of replication from pWSK29. The resulting low-copy number baculovirus transfer vector was designated pBacPAK9_{low}, and was verified by DNA agarose gel analysis.

The EE tag (Glu-Glu epitope tag) [26] was introduced at the 5'-end of the MNK cDNA by PCR using pCMB19 [24] as a

template and the mutagenic oligonucleotide 5'-CCAAATCTAG-AGGCCGCCACCATGGAGTTCATGCCGATGGAAGGAGG-ACCAAGTATGGGTGTGAATTCTGTACC-3'. The resulting N-terminal amino acid sequence of MNK was MEFMPME-GGPSMGV (the EE tag sequence is underlined with the three-residue linker in italics). The 0.9 kb PCR product was digested with XbaI and SphI, and exchanged with the corresponding XbaI-SphI fragment of MNK cDNA in pCMB19. The full-length EE-MNK (EE-tagged MNK) cDNA was subcloned into pBacPAK9_{low}. The presence of the additional EE tag was confirmed by DNA sequencing (Australian Genome Research Facility, Melbourne, VIC, Australia).

The low-copy number baculovirus transfer vector, pBacPAK9_{low} containing EE-MNK cDNA was co-transfected with BacPAK6 linearized baculovirus DNA (Clontech) into Sf9 cells using the Bacfectin® liposomal preparation according to the manufacturer's instructions (Clontech). The resulting recombinant baculovirus was purified by plaque assay and amplified as previously described [27].

Sf9 cell culture and baculovirus infection

Sf9 cells were grown as suspension cultures at cell densities in the range $(0.2-2) \times 10^6$ cells/ml. Small-scale cultures of Sf9 cells with volumes up to 250 ml were maintained in roller bottles in Grace's medium (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 0.3% (w/v) tryptose phosphate broth, 0.04% NaHCO₃ and 10% (v/v) fetal calf serum (Commonwealth Serum Laboratories, Melbourne, VIC, Australia) at 27°C. Large-scale cultures up to 6 litres for purification were carried out in a Bioflow 3000 bioreactor (New Brunswick Scientific, New Brunswick, NJ, U.S.A.) in Gibco® SF900 II serum-free medium (Invitrogen, Carlsbad) supplemented with 0.1% (v/v) Pluronic F-68 (Sigma-Aldrich, St Louis, MO, U.S.A.) at 27°C.

Sf9 cells were usually infected in the exponential growth phase at a cell density of $(1.5-2) \times 10^6$ cells/ml with the recombinant baculovirus at an MOI (multiplicity of infection) of 5 pfu (plaque-forming units)/ml. Cells were harvested between 50 and 60 h post-infection, and washed in ice-cold HB buffer (10 mM Tris/HCl, pH 7.4, 250 mM sucrose and 0.5 mM EDTA) supplemented with 1 mM DTT (dithiothreitol) and Roche Complete™ EDTA-free protease inhibitor tablet (Roche, Basel, Switzerland). Cell pellet was stored at -70°C until use.

Preparation of Sf9 crude membranes

Sf9 cell pellet was thawed and resuspended in ice-cold HB buffer supplemented with 1 mM DTT and Roche Complete™ EDTA-free protease inhibitor tablet. Cells were homogenized in a Dounce homogenizer (Braun, Melsungen, Germany), 20 strokes at 1200 rev./min. The homogenate was centrifuged at 10000 g for 10 min at 4°C. The soluble fraction (supernatant) was collected and further centrifuged for 60 min at 180000 g. The pelleted cell membranes were resuspended in HB buffer supplemented with 0.5 mM DTT and Roche Complete™ EDTA-free protease inhibitor tablet (Roche), then stored at -70°C until use.

Differential detergent solubilization of Sf9 membranes

EE-MNK-containing membranes were resuspended in ice-cold solubilization buffer [50 mM Mops/Tris, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 2 mM 2ME (2-mercaptoethanol) and Roche Complete™ EDTA-free protease inhibitor tablet] supplemented with DDM (*n*-dodecyl β -D-maltopyranoside), OG (*n*-octyl β -D-glucopyranoside), or TX100 (Triton X-100). Optimal solubilization conditions were determined for each detergent as

described in the Results section. The suspension was mixed and incubated at 4°C overnight. The soluble membrane protein fraction was collected by centrifugation at 20000 g for 20 min at 4°C.

Purification of MNK from Sf9 membranes

Small-scale protein purification to optimize conditions was carried out by immunoprecipitation. Soluble membrane protein fractions prepared as described above and EE-MNK were immunoprecipitated with 5–10 μ g of monoclonal anti-EE tag antibody (Monoclonal Antibody Facility, Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia) overnight at 4°C with mixing. The immunocomplex was recovered by incubation with Protein G Plus-agarose suspension according to the manufacturer's instructions (Calbiochem, San Diego, CA, U.S.A.). Unbound proteins were removed by washes in the solubilization buffer. EE-MNK was eluted with the solubilization buffer containing 50 μ g/ml EYMPME peptide (AusPep, Melbourne, VIC, Australia). The antibody recognizes the sequences EYMPME and EFMPME.

Large-scale protein purification was carried out by antibody affinity chromatography followed by size-exclusion chromatography. The soluble membrane protein fraction was applied to a 20 cm $\times$ 0.5 cm column containing monoclonal anti-EE tag antibody [26] covalently conjugated to Protein G-Sepharose Fast Flow using dimethylpimelimidate [28] at a concentration of 1–2 mg of antibody per ml of gel. The column was equilibrated with 50 mM Mops/Tris buffer (pH 7.5) containing 150 mM NaCl, 10% glycerol, 2 mM DDM and 2 mM 2ME, at a flow rate of 4 ml/min. The column was washed with 10 bed volumes of the same buffer. EE-MNK was eluted with a 50 ml linear gradient to 50 μ g/ml EYMPME peptide in the same buffer. Fractions of 2 ml were collected at a flow rate of 3 ml/min. The collected fractions were analysed by SDS/PAGE and visualized by Coomassie Brilliant Blue staining. Fractions containing EE-MNK were pooled and concentrated using an Amicon Ultra-15 PLGC centrifugal filter unit (Millipore, Billerica, MA, U.S.A.) with MWCO (molecular weight cutoff) 10000. EE-MNK was further purified by size-exclusion chromatography using a Superose-12 10/30 HR column (Amersham Biosciences, Uppsala, Sweden) equilibrated and developed in 50 mM Mops/Tris buffer (pH 7.5) containing 150 mM NaCl, 10% glycerol, 2 mM DDM, and 2 mM 2ME. Protein samples were eluted isocratically in the same buffer at a flow rate of 0.5 ml/min. Fractions were collected manually, analysed by SDS/PAGE and visualized by Coomassie Brilliant Blue staining. EE-MNK-positive fractions were pooled and concentrated as above. Purified EE-MNK protein was snap-frozen in liquid nitrogen and stored at -70°C until use. Identity of the purified protein sample was verified by tandem MS analysis of an in-gel tryptic digest of one-dimensional SDS/PAGE resolved EE-MNK (Joint Proteomics Service Facility, Ludwig Institute of Cancer Research and Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia).

Membrane reconstitution of MNK

Soya-bean asolectin (BioChemica, Melbourne, FL, U.S.A.) mixture of phospholipids was dissolved in chloroform and evaporated to dryness under a gentle stream of nitrogen gas. This was followed by vacuum drying in a desiccator overnight to ensure total removal of the organic solvent. The lipids were dispersed with degassed 50 mM Mops/Tris buffer (pH 7.5) containing 150 mM NaCl to the final concentration of 5 mg/ml. LUV (large unilamellar vesicles) were prepared by extruding the lipid solution through a 200 nm polycarbonate membrane using an Avanti® mini-extruder (Avanti Polar Lipids, Alabaster, AL, U.S.A.).

The purified EE-MNK protein was reconstituted into pre-formed liposomes by the method of detergent removal [29,30]. The purified protein, asolectin LUV and DDM in the reconstitution mixture were present in a 1:25:250 ratio (by wt.), and were incubated at room temperature (25 °C) for 2 h with gentle mixing. Detergent was removed by incubation with polystyrene beads, Bio-Beads SM-2 (Bio-Rad, Hercules, CA, U.S.A.), at a Bio-Beads to detergent ratio of 60, for 30 min at room temperature with gentle mixing. Bio-Beads incubation was repeated once before the beads were removed by sedimentation. Proteoliposomes containing EE-MNK were collected by centrifugation at 180 000 *g* for 60 min at 4 °C. Proteoliposomes were resuspended in 50 mM Mops/Tris buffer (pH 7.5) containing 150 mM NaCl, 10 % glycerol and 2 mM 2ME, and stored at -70 °C until use.

SDS/PAGE and Western-blot analysis

Protein samples were separated by SDS/PAGE using 4–12 % Bis-Tris gradient gels (Invitrogen, Carlsbad). Total protein was visualized by Coomassie Brilliant Blue staining. For Western-blot analysis, proteins were transferred on to a nitrocellulose membrane after separation by SDS/PAGE. EE-MNK was detected with antisera against the MNK N- or C-terminus [31,32], or the monoclonal anti-EE tag antibody. For protein detection by chemiluminescence, secondary IgG-HRP (horseradish peroxidase) conjugated antibodies (Silenus Labs, Melbourne, VIC, Australia) were used. Proteins were visualized using Lumi-Light Western blotting substrates (Roche) according to the manufacturer's instructions, and the blots were imaged using a Typhoon 8600 PhosphorImager (Molecular Dynamics).

Detection of the acyl-phosphate intermediate

Detection of MNK acyl-phosphate intermediate in the crude membrane preparations was performed as previously described [20]. The acyl-phosphate intermediate of the purified EE-MNK protein was detected using the method described in [33]. Radioactive [γ -³²P]ATP was purchased from PerkinElmer Life Sciences (Melbourne, VIC, Australia). Various assay conditions, copper concentrations, metals and inhibitors were added as described in the Figure legends. Samples were separated by SDS/PAGE on an acidic 5.5 % polyacrylamide gel. The radiolabelled MNK acyl-phosphate intermediate was visualized by exposing the dried gel to a phosphor screen for 3–24 h, and imaged with a Typhoon 8600 PhosphorImager (Molecular Dynamics). The acyl-phosphorylation activity was determined by normalizing the radiolabelled signal against the EE-MNK protein level.

ATPase assay

Purified EE-MNK solubilized in DDM was assayed for ATPase activity using a modified colorimetric method previously described [34]. Reactions were carried out in 20 mM Mops buffer (pH 6.8) containing 150 mM NaCl, 5 mM MgCl₂, 10 % glycerol and 2 mM DDM at 37 °C. Various copper concentrations were added as indicated. Reactions were initiated by the addition of 1 mM ATP. Samples containing 50 ng of EE-MNK were taken at 10 min intervals up to 60 min. Reactions were terminated by the addition of 50 μ l of 0.5 M EDTA. Free P_i was assessed by adding 100 μ l of Malachite Green solution A [0.034 % Malachite Green in 10 mM ammonium molybdate/1 M HCl/3.4 % (v/v) ethanol] and solution B (1 % Tween 20) mixed in equal parts. This was then incubated for 15 min at room temperature to allow colour development. Absorbance was measured at 650 nm using a PowerWave™ XS microplate spectrophotometer (Bio-Tek Instruments, Winooski, VA, U.S.A.). The amount of P_i released

was determined against a standard curve using KH₂PO₄. The rate of P_i released is expressed as nmol of P_i released · mg of protein⁻¹ · min⁻¹.

Copper transport assay

⁶⁴Cu-transport experiments were carried out using crude membrane preparations as described previously [22] and reconstituted EE-MNK proteoliposome. Radioactive ⁶⁴CuCl₂ was obtained from Australian Radioisotopes [the trading name of ANSTO (Australian Nuclear Science and Technology Organisation)] (Lucas Heights, NSW, Australia). Radioactivity was measured using an LKB-Wallac 1282 Compugamma Universal Gamma Counter (LKB Wallac, Turku, Finland).

Data analysis

Curves were fitted using GraphPad (San Diego, CA, U.S.A.; <http://www.graphpad.com>) Prism™ Software for Windows. Kinetic data were fitted using either the sigmoidal dose-response (variable slope) equation, or the classical Michaelis-Menten equation. In cases where the purified enzyme activity was inhibited at copper concentrations greater than 1 μ M, data points beyond that showing the maximum activity were excluded from the analysis.

RESULTS

High levels of baculovirus-mediated EE-MNK expression in Sf9 cells

Detailed biochemical characterization of the MNK copper transporting P-type ATPase is not currently possible due to low levels of endogenous and transfected expression of these proteins in mammalian cells. Higher heterologous MNK protein expression in *S. cerevisiae* could be obtained compared with mammalian cells, but the yeast-expressed protein had a smaller apparent molecular mass that was attributed to differential post-translational modifications [20]. Post-translational modifications in insect cells are similar to that of mammalian cells [27], and functional heterologous protein expression at high levels had been reported for other members of the P-type ATPase family [35–37]. Therefore, to obtain high functional expression of MNK copper-transporting P-type ATPase for purification, we expressed MNK with an N-terminal six-mer EE tag [26] in Sf9 cells using a baculovirus expression system. The additional EE tag was introduced to facilitate downstream protein purification.

EE-MNK protein expression in Sf9 cells was compared with the endogenous and transfected MNK protein levels in CHO-K1 cells (Chinese-hamster ovary K1 cells). The expression of MNK in Sf9 cells infected with the recombinant EE-MNK baculovirus at MOI of 1 pfu/ml was 65-fold higher than the endogenous MNK in CHO-K1 cells, and 4-fold higher than the transfected expression of MNK in CHO-K1 cells (Figure 2A). Infection of Sf9 cells with EE-MNK recombinant baculovirus at a higher MOI resulted in a corresponding increase in protein expression (results not shown). Western-blot analysis demonstrated that the recombinant EE-MNK protein was recognized by both MNK N- and C-terminal antisera, and the anti-EE tag antibody, indicative of a full-length protein expression.

The expression of EE-MNK in Sf9 cells was time-dependent (Figure 2B). However, a corresponding decrease in cell viability approx. 60 h post-infection was observed (results not shown), which could lead to increased heterogeneity in protein post-translational modifications and degradation. Therefore we

Figure 2 Baculovirus-mediated expression of EE-MNK in Sf9 cells

(A) Western-blot analysis comparing the expression of endogenous MNK protein in CHO-K1 cells, CHO-K1 cells overexpressing MNK, baculovirus-mediated expression of EE-MNK in Sf9 cells (MOI = 1), and endogenous MNK in Sf9 cells. For each sample 20 μ g of total protein extract was loaded. MNK was visualized by enhanced chemiluminescence following immuno-detection using an anti-MNK N-terminus (1:2500), anti-MNK C-terminus (1:2000), or anti-EE tag (1:250) antibodies, and secondary IgG-HRP conjugated antibodies (1:1500). (B) Time-dependent expression of EE-MNK protein. Sf9 cells infected with EE-MNK recombinant baculovirus at MOI = 1 were harvested at the indicated times post-infection. For each sample 50 μ g of total protein extract was loaded. EE-MNK was immuno-detected using anti-EE epitope tag antibody.

routinely infected Sf9 cells at MOI of 5 pfu/ml and harvested the cells 50–60 h post-infection.

EE-MNK in membrane vesicles is biochemically active

Characteristic of P-type ATPases is the formation of a vanadate-sensitive transient acyl-phosphate intermediate during their catalytic cycle (Figure 1B). Using membrane vesicles isolated from EE-MNK-expressing Sf9 cells and [γ - 32 P]ATP, we demonstrated the formation of the EE-MNK phosphoenzyme (Figure 3A). Increase in γ - 32 P labelling time was paralleled by an increase in EE-MNK phosphoenzyme formation. The turnover of the EE-MNK acyl-phosphate intermediate was rapid when pulse-chased with 1 mM unlabelled ATP. No detectable acyl-phosphorylation was observed in the uninfected Sf9 membrane vesicle control, or in the presence of 1 mM BCS (bathocuproinedisulfonic acid), a Cu(I) chelator, suggesting Cu(I) was an essential substrate for active EE-MNK acyl-phosphorylation.

Next, we assayed the vectorial copper transport by EE-MNK in membrane vesicles using radiolabelled 64 CuCl₂. Copper-activated vectorial 64 Cu transport of EE-MNK has an apparent $V_{\max}$ of 1.5 ± 0.03 nmol of Cu $\cdot$ mg of protein⁻¹ $\cdot$ min⁻¹, with an apparent $K_{0.5}$ of 1.1 ± 0.04 μ M (Figure 3B). The Hill coefficient (h) value of 2.3 ± 0.2 suggests positive co-operativity in copper binding. It should be noted that there was a decrease in 64 Cu-transport activity at copper concentrations above 3 μ M. This was likely to be the result of copper inhibition, consistent with that previously reported for MNK expressed in *S. cerevisiae* [20]. Data points beyond 3 μ M were excluded in the data analysis.

The ATP-dependent vectorial 64 Cu-transport by EE-MNK in Sf9 membrane vesicles follows the classical Michaelis-Menten equation (Figure 3C). The apparent K_m of EE-MNK for ATP was 3.6 ± 1.0 μ M. It was reported previously that the apparent K_m of MNK expressed in *S. cerevisiae* for ATP was 17 μ M [20]. In comparison, the apparent affinity of EE-MNK expressed in Sf9 cells was approx. 5-fold higher.

Figure 3 Biochemical properties of the recombinant EE-MNK expressed in Sf9 cells

Membrane vesicles isolated from Sf9 cells as described in the Experimental section were used in these experiments. (A) Cu(I)-dependent acyl-phosphorylation of EE-MNK. Twenty micrograms of total membrane protein from either Sf9 cells or EE-MNK protein-expressing Sf9 cells was labelled with [γ - 32 P]ATP and pulse-chased with 1 mM unlabelled ATP as described in [20]. Cu(I) was chelated by treatment with 1 mM BCS, a Cu(I) chelator. (B) Cu(I)-dependent 64 Cu-transport activity of EE-MNK. 64 Cu-transport activities for various copper concentrations were determined and data were fitted as described in the Experimental section. (C) ATP-dependent 64 Cu-transport activity of EE-MNK. (D) Inhibition of EE-MNK 64 Cu-transport by vanadate.

Vanadate, a structural homologue of inorganic phosphate, is a classical inhibitor of P-type ATPases. The binding of vanadate to the conserved aspartic acid residue prevents the formation of the transient acyl-phosphate intermediate [12]. The IC₅₀ of vanadate for vectorial 64 Cu-transport of EE-MNK in Sf9 membrane vesicles was 81.1 ± 0.1 μ M (Figure 3D), consistent with previous findings that the heavy metal P-type ATPases are generally less sensitive to vanadate inhibition compared with non-heavy metal P-type ATPases [20,22,33,38]. Taken together, these results demonstrated that the EE-MNK protein expressed in Sf9 cells was a functional copper transporting P-type ATPase.

Differential detergent solubilization of EE-MNK

Detergents are central to the isolation and solubilization of membrane proteins, and the selection of suitable detergent(s) is an important aspect of successful functional membrane protein purification. The non-ionic detergents DDM, OG and TX100 were found to be effective in functional purification and reconstitution of a number of P-type ATPases [33,39–41]. These detergents were compared for EE-MNK extraction efficiency and retention of enzymatic activity.

Complete and stable solubilization of membrane proteins generally occurs above the detergent CMC (critical micelle concentration). Here, we tested the solubilization of 200 μ g of total membrane protein with either DDM or TX100 at $10 \times$ CMC, or OG at $2 \times$ CMC (Figure 4A). It should be noted that both DDM and TX100 have a low CMC value, 0.2 and 0.3 mM respectively, whereas OG has a high CMC value of 23.2 mM. At the concentrations used, the percentage concentration for each detergent was within the typical membrane protein solubilizing detergent concentration of 0.1–2%.

The solubilized EE-MNK protein was recovered by immuno-precipitation and eluted using the EYMPME peptide. Under the conditions used in the present study, SDS/PAGE analysis of soluble membrane protein fractions and protein recovery after

Figure 4 Differential non-ionic detergent solubilization of recombinant EE-MNK protein from Sf9 membranes

(A) Total membrane proteins (200 µg) were solubilized (S) with 2 mM DDM, 46 mM OG or 3 mM TX100. EE-MNK was immunoprecipitated using the anti-EE epitope tag antibody and 15 µl of eluent (E) per sample was analysed by SDS/PAGE as described in the Experimental section. The arrow indicates the position of the 180 kDa EE-MNK protein. (B) Acyl-phosphorylation activity of EE-MNK in the presence of non-ionic detergents. Total membrane protein (20 µg) was labelled with [γ - 32 P]ATP for 20 s in the presence of DDM, OG or TX100 at various concentrations as indicated. Acyl-phosphorylation activity is expressed as percentage activity of the control non-solubilized Sf9 membrane preparation.

immunoprecipitation revealed that DDM and TX100 were most efficient in membrane protein solubilization with an equivalent EE-MNK protein recovery. However, membrane protein solubilization by OG was inefficient with poor protein recovery, which agrees with studies that found OG deleterious to the Ca²⁺-ATPase [41,42]. Furthermore, crude membranes solubilized with DDM demonstrated higher retention of EE-MNK acyl-phosphorylation activity compared with OG and TX100 (Figure 4B). In an additional screen, we found DDM at 10 mM to solubilize membrane proteins more efficiently than at 2 mM (results not shown). From these results, we decided to use DDM at 10 mM for membrane protein extraction, and to use 2 mM DDM for purification and storage of EE-MNK.

Purification of EE-MNK

For a typical large-scale purification of EE-MNK, membrane proteins were extracted from Sf9 crude membranes prepared from approx. 4.7×10^9 cells. Antibody affinity chromatography was used to capture EE-MNK from the 10 mM DDM soluble membrane protein fraction, followed by size-exclusion chromatography to remove the EYMPME peptide, contaminants and degraded protein fragments. However, as a result of protein loss during the purification process, relatively low amounts of purified

Figure 5 Purification of detergent-solubilized EE-MNK protein

Crude membranes isolated from Sf9 cells expressing EE-MNK were solubilized with 10 mM DDM, and the soluble fraction was collected by centrifugation at 20000 g for 20 min at 4 °C (membrane extract). EE-MNK was purified as described in the Experimental section. Collected fractions from the affinity and size-exclusion chromatography (SEC) columns were analysed by SDS/PAGE and concentrated (C). (A) A sample of 5 µg from each protein fraction and 5 µl of the purified EE-MNK protein were analysed by SDS/PAGE and visualized by Coomassie Brilliant Blue staining. Asterisk denotes EE-MNK in the crude protein samples. (B) Western-blot analysis of the purified EE-MNK protein detected with the anti-MNK N-terminus antiserum (1:2500). Per lane, 50–100 ng of the purified EE-MNK protein was loaded.

EE-MNK, approx. 185 µg, were recovered. The purified EE-MNK was assessed to be purified to homogeneity by Coomassie Brilliant Blue-stained SDS/PAGE (Figure 5A). The protein identity was confirmed by Western-blot analysis (Figure 5B) and tandem MS analysis (results not shown).

Detergent-solubilized EE-MNK is catalytically active

Catalytically active purified EE-MNK solubilized in 2 mM DDM was demonstrated by acyl-phosphorylation analysis. Compared with EE-MNK in Sf9 membrane vesicles (Figure 3A), purified EE-MNK solubilized in 2 mM DDM required a longer labelling time (15 min) before maximal acyl-phosphorylation was achieved (Figure 6A). When pulse-chased with 1 mM unlabelled ATP, the turnover of the acyl-phosphate intermediate was also slower after the initial rapid decrease in radiolabelled phosphoenzyme compared with that in Sf9 membrane vesicles. This result suggests that lipids may play a role in EE-MNK catalytic activity. The acyl-phosphorylation activity of purified EE-MNK was abolished in the presence of 1 mM BCS, which agrees with previous findings that Cu(I) is required for MNK activation [20,22,23]. Contrary with expectations, the acyl-phosphorylation activity was relatively high in the absence of added reductant, DTT, which was likely to be due to an approx. 0.2 mM residual 2ME carried over from the storage/elution buffer (Figure 6B). This presumably provided sufficient reducing conditions for CuCl₂ to be reduced to Cu(I).

To assess metal specificity of the purified EE-MNK, we assayed for its phosphoenzyme formation in the presence of 1 µM copper, silver, zinc or cadmium. Based on previous metal specificity studies involving MNK [6,20], it was expected that the univalent metal ion Ag(I), which is chemically similar to Cu(I), would be able to activate the purified EE-MNK phosphoenzyme formation, but not the bivalent Zn(II) or Cd(II). In agreement with previous studies [6,43], Ag(I)-stimulated purified EE-MNK acyl-phosphorylation activity was approx. 80% that for Cu(I).

Figure 6 Analysis of the purified EE-MNK protein acyl-phosphorylation activity

Purified EE-MNK protein (250 ng) solubilized in 2 mM DDM was assayed for acyl-phosphorylation activity as described in the Experimental section. **(A)** The acyl-phosphate intermediate formation by the purified EE-MNK protein was time-dependent and transient. Purified EE-MNK protein was labelled with [γ - 32 P]ATP for up to 15 min as indicated. The pulse-chase experiments using 1 mM unlabelled ATP were performed following 15 min labelling. **(B)** Cu(I)-dependent EE-MNK protein acyl-phosphorylation. Purified EE-MNK protein was assayed for the formation of the acyl-phosphate intermediate in the absence of a reductant, DTT, and in the presence of the Cu(I) chelator, BCS, at 0.5 and 1 mM concentrations. **(C)** Metal specificity of the purified EE-MNK protein. Purified EE-MNK was assayed for acyl-phosphate intermediate formation in the presence of 1 μ M Cu(I), Ag(I), Zn(II) or Cd(II). DTT (50 μ M) was included in the Cu(I) assay mixture. **(D)** Effect of inhibitors on purified EE-MNK protein acyl-phosphorylation activity. Purified EE-MNK protein was pre-incubated with 0.5 mM sodium orthovanadate (Va) or 1 mM NEM. Treatment with hydroxylamine (HA) was performed after trichloroacetic acid precipitation by resuspending the protein pellet in 200 μ l of 0.1 M sodium acetate (pH 5.6), followed by incubation with 0.2 ml of 0.25 M hydroxylamine at room temperature for 10 min, and re-precipitation with trichloroacetic acid. KOH treatment of the purified EE-MNK protein acyl-phosphate intermediate was carried out by resuspending the trichloroacetic acid-precipitated protein pellet with 0.2 ml of 0.5 M KOH, and incubation on ice for 5 min before re-precipitation with trichloroacetic acid. **(E)** Cu(I)-dependent acyl-phosphorylation activity of the purified EE-MNK protein.

Figure 7 Copper-dependent ATPase activity of EE-MNK

Purified EE-MNK protein solubilized in 2 mM DDM was assayed for ATPase activity as described in the Experimental section.

In contrast with previous reports [20], Zn(II) was also able to stimulate considerable EE-MNK phosphoenzyme formation (~60%).

Formation of the acyl-phosphate intermediate by purified EE-MNK was 80% inhibited by the P-type ATPase inhibitor, vanadate (Figure 6D). When pre-incubated with NEM (*N*-ethylmaleimide), an alkylator of solvent-accessible thiol groups, acyl-phosphorylation activity of purified EE-MNK was significantly inhibited. This suggested that in the solubilized state, the cysteine residues that are proposed to be involved in copper coordination and Cu(I)-dependent acyl-phosphorylation were readily accessible. Furthermore, the purified EE-MNK phosphoenzyme was only stable under acidic conditions, which was abolished after KOH treatment (Figure 6D). The presence of the acyl-phosphate bond in the purified EE-MNK phosphoenzyme was further demonstrated by its sensitivity to hydroxylamine, which cleaves the acyl-phosphate bond (Figure 6D).

The Cu(I)-activated acyl-phosphorylation (Figure 6E) and ATPase (Figure 7) activities of purified EE-MNK were fitted to a sigmoidal function. These results further supported our data on copper-dependent vectorial ^{64}Cu transport by EE-MNK in Sf9 membrane vesicles (Figure 3B), which also indicated that copper ions bind to EE-MNK in a co-operative manner. The EC_{50} for copper-dependent acyl-phosphorylation of purified EE-MNK was $0.7 \pm 0.03 \mu\text{M}$, with an h value of 4.6 ± 0.1 . In the absence of contaminating ATPases, we were able to assay MNK ATPase activity for the first time, and the $K_{0.5}$ for copper-dependent ATPase activity was determined to be $0.6 \pm 0.04 \mu\text{M}$, with an h value of 5.0 ± 2.4 .

Active vectorial copper transport by reconstituted EE-MNK

An impermeable membrane barrier is required to study vectorial copper transport using radiolabelled $^{64}\text{CuCl}_2$. In order to measure vectorial copper transport activity of purified EE-MNK, we reconstituted it into phospholipid liposomes. Soya-bean asolectin phospholipid mix, which has been used successfully for the reconstitution of the *E. hirae* CopA copper-transporting P-type ATPase [33] and the Ca^{2+} -ATPase [44], was used as a source of phospholipids. The purified EE-MNK protein was reconstituted into preformed soya-bean asolectin liposomes by the method of detergent removal using polystyrene beads, Bio-Beads SM-2 [29,30]. A lipid to protein ratio (w/w) of 25 was used in this experiment, which was determined by comparing the acyl-

Figure 8 Active ^{64}Cu transport by reconstituted EE-MNK protein

Purified EE-MNK protein was reconstituted into preformed soya-bean asolectin liposomes as described in the Experimental section, and ^{64}Cu -transport experiments were performed in the presence of 20 nM $^{64}\text{CuCl}_2$. (A) The ^{64}Cu -transport activity of reconstituted EE-MNK was ATP-dependent. ^{64}Cu -transport activity was measured in the absence of ATP (■), or in the presence of 5 mM ATP (▲). (B) Comparison of the ATP-dependent copper transport activity between the empty asolectin liposome, reconstituted EE-MNK proteoliposome, and EE-MNK in Sf9 membrane vesicles.

phosphorylation activity of purified EE-MNK with the soya-bean asolectin lipids present at different mass ratios (results not shown). Typically, protein recovery after reconstitution was approx. 5% as assessed by protein assay.

Translocation of ^{64}Cu mediated by EE-MNK into reconstituted proteoliposomes was inhibited at high copper concentrations, and we were only able to measure EE-MNK ^{64}Cu -translocation activity when nanomolar amounts of copper were used. The ATP-dependent ^{64}Cu -transport specific activity of membrane reconstituted EE-MNK was $44.9 \text{ pmol of Cu} \cdot \text{mg of protein}^{-1} \cdot \text{min}^{-1}$ (Figure 8A). Under the present experimental conditions, the reconstituted EE-MNK had increased approx. 17-fold in specific activity compared with that in Sf9 membrane vesicles

(Figure 8B). This result demonstrated that the purified EE-MNK was able to actively transport copper, and suggests that most of the purified EE-MNK was incorporated into preformed liposomes in the desired inside-out orientation with an external presentation of catalytic domains.

DISCUSSION

The P1_B-subfamily of heavy metal transporting P-type ATPases has stimulated many studies on metal-ion homeostasis and heavy metal detoxification. Currently, the well-characterized Ca²⁺-ATPase provides the basis for understanding the structure and mechanism of the heavy metal transporting P-type ATPases. Detailed structure and functional studies of the mammalian heavy metal transporting P-type ATPases have been impeded by technical difficulties in the purification of these multi-spanning membrane proteins in an active form. In the present study, we report the first direct catalytic analysis of a purified and membrane reconstituted full-length MNK copper-transporting P-type ATPase, an essential copper homeostasis component in humans.

One of the limitations for achieving functional purification of membrane proteins is the availability of the protein in the starting material in sufficiently high yields. The baculovirus expression system enabled us to achieve high levels of recombinant EE-MNK protein expression compared with that expressed in mammalian cells. EE-MNK in membrane vesicles isolated from Sf9 cells exhibited higher copper transport activity compared with that previously reported for MNK expressed in mammalian and yeast cells [20], and this is consistent with the higher MNK expression in Sf9 cells. The biochemical data obtained in the present study using membrane vesicles agree with previous functional studies of MNK [20,22], and validate the proposed function of MNK as a Cu(I)-transporting P-type ATPase.

In addition to Cu(I) activation of MNK, Ag(I) and Zn(II) ions were also able to activate the formation of the acyl-phosphate intermediate, but currently there is no experimental data to show that they are also transported across the membrane bilayer by MNK. Ag(I) transport has been demonstrated for the prokaryotic copper-transporting homologues of MNK, CopB from *E. hirae* [43] and CopA from *Archaeoglobus fulgidus* [45], but with much lower affinity for Ag(I) compared with Cu(I). Thus the ability of the univalent Ag(I) to activate the purified EE-MNK acyl-phosphorylation is not unusual, given its chemical similarity to Cu(I).

Zn(II)-stimulated formation of the purified EE-MNK phosphoenzyme was a novel observation. Studies by DiDonato et al. [46,47] investigating the metal binding properties of the isolated N-terminal metal binding domain of WND reported the ability of this domain to bind a number of bivalent cations, including Zn(II). Replacement of the N-terminal metal binding domain of *E. coli* ZntA P-type ATPase, which has substrate specificity for Cd(II), Pb(II) and Zn(II), with that from WND did not confer Cu(I) and Ag(I) substrate specificity [48]. Therefore metal specificity is not determined by the N-terminal metal binding domain of heavy metal transporting P-type ATPases, but lies within the transmembrane channel metal binding site(s). It is possible that the protein conformation adopted by purified EE-MNK solubilized in DDM may differ from the membrane-embedded protein, thus allowing Zn(II) ions to bind and stimulate the E₁-P phosphoenzyme formation, but unlikely to proceed beyond this step. Currently, we are not aware of any experimental evidence of vectorial Zn(II) transport by either of the mammalian copper-transporting P-type ATPases. *In vivo*, correct metal transport catalysed by metal-ion transporting ATPases is strongly regulated

by interactions with associated metallochaperones involved in metal trafficking pathways [49].

ATP hydrolysis activity of MNK, a key step in the proposed catalytic cycle, was demonstrated in the present study for the first time. Measurements of MNK ATPase activity had not been possible previously due to high background of contaminating ATPases present in membrane vesicles isolated from mammalian and yeast cells. The demonstration of ATP hydrolysis by purified MNK further validates its classification as a Cu(I)-transporting P-type ATPase. Portmann and Solioz [50] recently reported the purification and reconstitution of a truncated WND without the first five MBSs ($\Delta 1-5$ WND), which has a maximal Cu(I)-dependent ATPase activity of 7–8 nmol of P_i released · mg of protein⁻¹ · min⁻¹ in the presence of added 10 μ M Cu(I). In comparison, the purified full-length MNK has a $V_{\max}$ of 56 nmol of P_i released · mg of protein⁻¹ · min⁻¹ in the presence of added 1 μ M Cu(I). These results suggest that an intact N-terminal metal binding domain is likely to be essential for optimal ATPase activity.

It is noteworthy that auxiliary protein cofactors are not required for purified EE-MNK catalytic activity. The metallochaperone Atox1 [ATX1 antioxidant protein 1 homologue (yeast)], essential for copper delivery to MNK and copper-dependent trafficking of MNK [51], has been reported to stimulate the activity of WND [52]. It is therefore possible that copper loaded Atox1 can also stimulate the catalytic activity of purified EE-MNK. Also, in the absence of membrane lipids, the acyl-phosphorylation activity of purified EE-MNK solubilized in DDM was considerably lower than that in Sf9 membrane vesicles. This result suggests that membrane lipids are required to enhance protein activity, and partial protein inactivation by the detergent cannot be discounted for the reduction in enzymatic activity. The effect of lipids and Atox1 on the purified MNK catalytic activity will form the basis of future studies.

Kinetic analysis of EE-MNK in Sf9 membrane vesicles and purified EE-MNK with respect to copper dependence revealed that it interacts with copper co-operatively with high affinity. The apparent affinity of EE-MNK in Sf9 membrane vesicles for copper was 1.1 μ M, approx. 4- and 2-fold higher than that previously determined for MNK expressed in mammalian cells (4 μ M) [23], and yeast cells (2 μ M) [20] respectively. It is interesting to note that in a recent study by Barnes et al. [53], the copper affinity for MNK similarly expressed in Sf9 cells is reported to be slightly lower at 2.4 μ M. This discrepancy may be the result of different experimental methods. In the present study, the affinity of EE-MNK in Sf9 membrane vesicles for copper was determined by ⁶⁴Cu-transport assay, whereas the result in the study of Barnes et al. [53] was based on the acyl-phosphorylation activity. However, the affinity of EE-MNK in Sf9 membrane vesicles for copper was comparable with that determined for WND (1.1 μ M) [53], CopA from *E. coli* (1.5 μ M) [54], and CopB from *E. hirae* (1 μ M) [43]. When purified, EE-MNK has an increased affinity for copper, which was determined to be in the sub-micromolar range (0.6 μ M). Compared with the purified CopA from *A. fulgidus* (2.1 μ M) [45], the purified EE-MNK protein has a 3- to 4-fold higher affinity for copper. The copper dependence data in the present study indicated co-operative interactions between MNK and copper that were not previously observed in our studies with the protein expressed in mammalian and yeast cells, but is corroborated by two recent studies of WND and MNK proteins expressed in Sf9 cells [37,53]. This result is consistent with copper-binding studies with isolated MNK N-terminal copper binding domains [55,56]. Taken together, these data reflect the regulatory role of MNK in cellular copper homeostasis. Co-operativity between copper binding sites may be

important in the induction of MNK conformational changes that lead to the trafficking of MNK from TGN to the plasma membrane where MNK mediates the efflux of excessive copper [6].

We have presented here the first experimental evidence of a functional reconstitution and direct measurement of vectorial copper translocation by a mammalian copper P-type ATPase. Active copper transport measured using the EE–MNK reconstituted proteoliposomes was indicative of effective incorporation of EE–MNK in the desired inside-out orientation into preformed soya-bean asolectin liposomes. However, it was beyond the scope of the present study to characterize the protein–lipid interactions of the reconstituted EE–MNK protein in detail. Nevertheless, this experiment is a key advance in establishing an *in vitro* membrane model, which will enable us to reconstitute the intracellular copper transport pathway by further biochemical and biophysical analyses.

Protein–lipid interactions are central to many cellular processes such as signalling and membrane trafficking. Future investigations using a model membrane system together with *in vivo* protein trafficking studies will enable an understanding of the influence of lipids on MNK catalytic and trafficking properties. In addition, the availability of purified MNK will enable structural studies, including comparisons with SERCA1a, and elucidation of the proposed linkage between conformational state in the catalytic cycle and copper-responsive trafficking of MNK [57,58].

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