Copper stimulates trafficking of a distinct pool of the Menkes copper ATPase (ATP7A) to the plasma membrane and diverts it into a rapid recycling pool

Luke PASE[1], Ilia VOSKOBOINIK[1,2], Mark GREENOUGH and James CAMAKARIS[3]

Department of Genetics, University of Melbourne, Melbourne, VIC 3010, Australia

MNK (Menkes copper-translocating P-type ATPase, or the Menkes protein; ATP7A) plays a key role in regulating copper homoeostasis in humans. MNK has been shown to have a dual role in the cell: it delivers copper to cuproenzymes in the Golgi compartment and effluxes excess copper from the cell. These roles can be achieved through copper-regulated trafficking of MNK. It has previously been shown to undergo trafficking from the *trans*-Golgi network to the plasma membrane in response to elevated copper concentrations, and to be endocytosed from the plasma membrane to the *trans*-Golgi network upon the removal of elevated copper. However, the fundamental question as to whether copper influences trafficking of MNK to or from the plasma membrane remained unanswered. In this study we utilized various methods of cell-surface biotinylation to attempt to resolve this issue. These studies suggest that copper induces trafficking of MNK to the plasma membrane but does not affect its rate of internalization from the plasma membrane. We also found that only a specific pool of MNK can traffic to the plasma membrane in response to elevated copper. Significantly, copper appeared to divert MNK into a fast-recycling pool and prevented it from recycling to the Golgi compartment, thus maintaining a high level of MNK in the proximity of the plasma membrane. These findings shed new light on the cell biology of MNK and the mechanism of copper homoeostasis in general.

Key words: copper, heavy metal, menkes copper ATPase (ATP7A), Menkes disease, trafficking.

INTRODUCTION

MNK (Menkes copper-translocating P-type ATPase, or the Menkes protein; ATP7A) is a ubiquitous copper-translocating P-type ATPase, which plays a crucial role in copper homoeostasis [1,2]. Mutations in the *ATP7A* gene may cause Menkes disease, which is usually a lethal X-linked recessive disorder in humans associated with systemic copper deficiency due to impaired absorption of alimentary copper from the gastrointestinal tract [3]. MNK is normally localized at the TGN (*trans*-Golgi network) of epithelial cells, where it supplies copper to copper-dependent enzymes in the secretory pathway [4,5], but it also recycles constitutively between the TGN and the PM (plasma membrane) [4,6]. Furthermore, in the presence of elevated extracellular copper concentrations MNK traffics to the PM, and upon the removal of excess copper it returns to the TGN [4]. Using the same cells as in the current study it had been shown previously that (i) copper-regulated trafficking is specific to univalent copper and silver ions, and (ii) MNK co-localizes with a TGN marker protein, p230, although p230 did not exhibit copper-induced trafficking to the PM where MNK co-localized with Na$^+$/K$^+$-ATPase [4]. The ability of MNK to undergo vesicular trafficking to the PM in response to elevated copper concentrations is believed to be responsible for systemic absorption of copper in the gut and its reabsorption in the kidney [4]. Relocalization of MNK to the PM also allows excess, potentially toxic, copper to be rapidly effluxed from cells [1]. The combination of copper-translocating catalytic activity with copper-dependent trafficking of MNK is pivotal for maintaining copper homoeostasis [1] and effects on one or both of these processes may lead to Menkes disease or occipital horn syndrome [7].

Several reports have indicated an important role for cytosolic motifs in copper-dependent trafficking of MNK. Among these are the N-terminal putative copper-binding sites, which are required for copper-dependent trafficking to the PM [8], the C-terminal di-leucine motif required for endocytosis [9] and the TGN retention motif [10]. Recently, a link between the catalytic activity and trafficking of MNK was established [11,12]. However, despite these findings, the detailed mechanism of copper-stimulated trafficking of MNK is unknown, primarily because of technical problems associated with overexpression of sufficient amounts of MNK in mammalian cells. In addressing the mechanism, it is important to determine (i) whether copper influences the rate of trafficking to and from the PM and (ii) whether there are distinct pools of MNK, which undergo copper-dependent trafficking.

In the current study we investigated the effects of copper on various stages of MNK trafficking using cell-surface biotinylation. We provide evidence that copper does not affect the rate of endocytosis from the PM, but appears to divert MNK-containing vesicles from the constitutive endocytic recycling via the TGN to a fast-recycling pool. Furthermore, copper was found to stimulate the recruitment of a distinct pool of MNK to traffic to the PM from the TGN.

EXPERIMENTAL

Cell culture and general methods

In this study we used copper-resistant variants of CHO-K1 cells (Chinese hamster ovary K1 cells), CUR2 and CUR3, which overexpress wild-type MNK [13]. In comparison, the parental CHO-K1 cells express very small amounts of MNK [13] and

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therefore are not suitable for current studies. In CUR2 and CUR3 cells, MNK displays normal copper-regulated trafficking [4] and biochemical properties [2,13]. Cells were cultured as a monolayer in Eagle's basal medium with Earle's salts (BME) supplemented with 10 % foetal bovine serum (Trace Biosciences, Noble Park, Victoria, Australia), 2 mM L-glutamine, 0.2 mM proline, 1.2 mM NaHCO$_3$ and 20 mM Hepes [13]. The copper concentration in this basal medium is 0.8 μM. All incubations were conducted at 37 °C. Biotin labelling, cleavage and processing of cell lysates were conducted at 4 °C. Unless indicated otherwise, CUR3 cells were used in all studies.

The cells were cultured in 30 mm Petri dishes. Cell-surface proteins were labelled for 30 min with 0.5 mg/ml cleavable water-soluble cell-impermeant sulpho-NHS-SS-biotin (Pierce Endogen) in PBS (pH 8.0) supplemented with 0.5 mM MgCl$_2$ and 1 mM CaCl$_2$. The unreacted NHS-SS-biotin was quenched by 100 mM glycine. Cleavage of the disulphide bridge of sulpho-NHS-SS-biotin for studying MNK endocytosis and recycling was achieved by incubating labelled cells with 100 mM GSH, which was subsequently quenched by 1 mg/ml iodoacetamide [14]. The efficiency of cleavage was approx. 95 %. The cells were lysed in 500 μl of lysis buffer containing 50 mM Tris (pH 7.5), 1 % Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mg/ml iodoacetamide and anti-protease cocktail (Roche Biochemicals), and precipitated on streptavidin–agarose beads (Pierce Endogen) overnight. The beads were washed three times in lysis buffer, twice in high-salt buffer (500 mM NaCl in 50 mM Tris, pH 7.5) and once in no-salt buffer (10 mM Tris, pH 7.5), and were eluted in loading buffer (Invitrogen) supplemented with 50 mM dithiothreitol. Proteins were resolved on 4–12 %-gradient SDS/PAGE gels (Invitrogen) and subjected to Western immunoblot analysis, where the MNK protein was detected using primary anti-MNK antibodies [13] and secondary horseradish-peroxidase-linked antibodies and developed using the chemiluminescence kit (Roche Biochemicals). The relative amounts of MNK were analysed by phosphorimaging using ImageQuant software (Typhoon 8600, Molecular Dynamics, Amersham Biosciences). Aliquots (5 μl) of cell lysate were retained prior to streptavidin precipitation. These samples were used for the Western analysis of the total MNK, and provided a measure of the relative amount of the total protein used for individual streptavidin precipitations. These values were subsequently used for standardization of streptavidin-precipitated MNK.

Radioactive ^{64}Cu uptake was conducted by adding 50, 100 or 500 μM ^{64}CuCl$_2$ (50 μCi/ml; ARI, Lucas Heights Research Laboratories, Lucas Heights, NSW, Australia) to the cells growing on 60 mm Petri dishes. At specific time points the cells were chilled on ice, copper was removed and cells were treated with Pronase as described in [13].

Intracellular localization of MNK in CUR3 cells following copper treatment was investigated using laser-scanning confocal immunofluorescence microscopy. The cells were subjected to copper treatment (500 μM copper) for various periods of time in order to study trafficking of MNK to the PM, or, following 3 h exposure of cells to 500 μM copper, basal medium was added to the cells for various periods of time in order to study endocytic trafficking of MNK. At specific time points the growth medium was removed, and the cells were washed with ice-cold PBS (pH 7.4) and then fixed with 4 % paraformaldehyde at room temperature for 15 min. The cells were washed several times with the PBS and permeabilized with 0.1 % Triton X-100 for 5 min. The cells were washed several times and blocked overnight with 1 % BSA (Sigma) in PBS prior to probing cells with the primary anti-MNK antisera [4] and the secondary anti-rabbit antibody Alexa 488 (Molecular Probes).

Experimental design

Rates of MNK trafficking to the PM in copper-resistant cells were estimated by culturing these cells in non-toxic concentrations of copper, up to 500 μM [13], for various periods of time prior to biotinylation, and were processed as described above.

Another type of study was conducted to estimate specifically the rate of appearance of a previously labelled (trafficking) pool of MNK at the PM. Cells were incubated in 500 μM copper for 3 h, biotinylated, and returned to basal medium for 2 h. Subsequently, the cells were again incubated with 500 μM copper for 5–90 min, and the cell-surface MNK–SS-biotin complex was cleaved with GSH prior to cell lysis (as described above). The non-cleaved, non-PM (internalized) intracellular pool of biotinylated MNK was detected by Western blot analysis using anti-MNK antibodies as described above. Intracellular reducing environment had no detectable effect on the stability of the internalized (intracellular) MNK–SS-biotin complex over at least a 2 h period (results not shown). This is consistent with the biotin-SS tag expected to be intraluminal and, therefore, not exposed to the strong reducing environment of the cytosol.

The rates of MNK endocytosis from the PM were estimated by culturing the cells in 500 μM extracellular copper for 3 h, and subsequent labelling with sulpho-NHS-SS-biotin. Cells were returned to culture medium without added copper or supplemented with 500 μM copper for 1–30 min. At the end of the incubation, the cell-surface biotin was cleaved with GSH, cells were lysed and internalized biotinylated proteins were precipitated using streptavidin and analysed as described above. In order to identify and study a postulated fast-recycling pool of MNK, the cells were exposed to 500 μM copper for 3 h, biotinylated and returned to the medium supplemented with 500 μM copper for 15 min. To eliminate the contribution of residual PM biotinylated MNK, surface biotin was subsequently cleaved with GSH. The cells were returned to 500 μM copper for 1–15 min and cell-surface biotinylated MNK was cleaved once again with GSH at given time points. The cells were subsequently lysed, processed, and biotinylated MNK was detected using Western blots as described above.

The apparent kinetic constants reported in this study were estimated by using non-linear regression analysis, which can be described by the equation $[\text{MNK}]_t = [\text{MNK}]_{t=0} + [\text{MNK}]_{max} \times (1 - e^{-kt})$, where k is the rate constant, t refers to individual time points, $[\text{MNK}]_{max}$ is the maximum theoretical level of MNK at the PM, and $[\text{MNK}]_{t=0}$ indicates the level of MNK at time 0. The $t_{1/2}$ values were calculated as $t_{1/2} = \ln 2/k$, and the initial rate values were estimated from the differentiated equation (see above).

RESULTS

Copper-dependent trafficking of MNK to the PM

In the current study, consistent with our original finding [4], we observed a time-dependent translocation of MNK in CUR3 cells from the TGN to the PM in the presence of 500 μM copper (Figure 1). However, despite pronounced relocalization of MNK to the PM, a significant proportion of MNK was also associated with vesicular structures throughout the cytosol (Figure 1 [4]). Also consistent with our earlier findings [4], upon the removal of elevated extracellular copper, the steady-state level of MNK at the PM started to diminish within several minutes, and by 60 min MNK appeared to return to its original steady-state location in the TGN (Figure 1). In order to investigate the role of copper in regulating trafficking of MNK to the PM and internalization from the PM (endocytosis), we applied the quantitative method of

Trafficking to the plasma membrane

Endocytosis

0

15min

30min

60min

120min

180 min

0

2 min

5 min
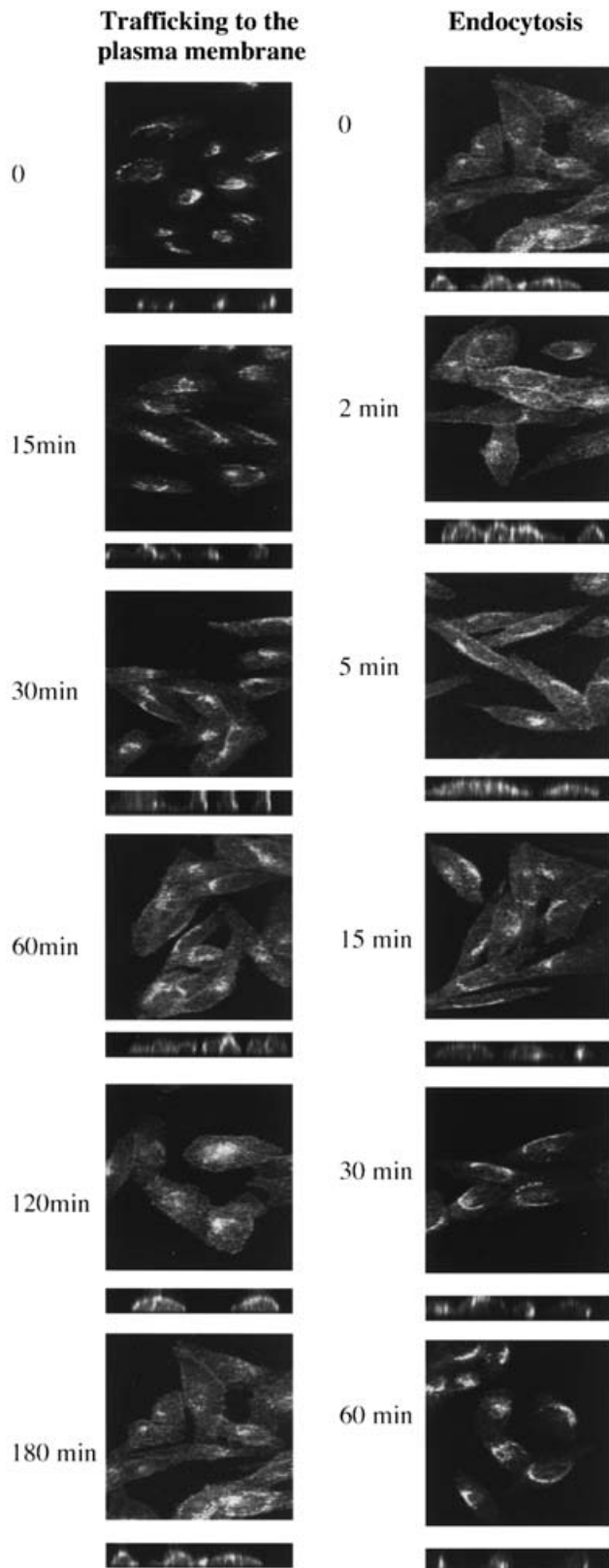
15 min

30 min

60 min

Figure 1   Intracellular localization of the MNK protein in CUR3 cells in the presence of elevated extracellular copper or after withdrawal from added copper medium

In experiments examining trafficking of MNK to the PM, the cells were grown in culture medium in the presence of 500 μM copper for 0–180 min prior to fixation. In endocytosis experiments

cell-surface protein biotinylation using the cell-impermeant reagent, sulpho-NHS-SS-biotin.

First, we investigated whether MNK could be biotinylated directly at the cell surface. Figure 2(A) shows detection of biotinylated MNK at the cell surface and demonstrates the expected increase following exposure of cells to elevated copper (500 μM). Furthermore, the results presented in Figure 2(A) provide strong evidence that all detectable biotinylated MNK was precipitated using streptavidin–agarose beads. The exposure of cells to a hyperosmotic environment, 0.45 M sucrose, resulted in the inhibition of MNK endocytosis, as determined by imaging studies [15]. As indicated by complete cleavage of the cell-surface biotinylated MNK with glutathione, MNK did not undergo any detectable internalization from the cell surface under hyperosmotic conditions. As a control for efficiency of biotinylation and streptavidin precipitation we analysed the predominantly PM-localized Na$^+$/K$^+$-ATPase. We found that $> 90\%$ of Na$^+$/K$^+$-ATPase was biotinylated and precipitated on streptavidin–agarose beads (L. Pase, I. Voskoboinik and J. Camakaris, unpublished work).

MNK relocalization to the PM was copper-concentration-dependent (Figure 2B), with the maximum steady-state level of MNK being observed following addition of 100 μM copper to the cells for 3 h. Under steady-state conditions there was a 4–5-fold increase in the amount of MNK at the PM following a 3 h exposure to 500 μM copper compared with basal copper conditions (Figures 2 and 3).

To determine whether the steady-state level of MNK at the PM is due to maintained elevated intracellular copper, we conducted time-dependent and concentration-dependent ^{64}Cu-uptake experiments (Figures 2B and 3). The data suggested that the maximum level of MNK at the PM coincided with accumulation of approx. 0.1 pmol of ^{64}Cu/μg of protein (Figure 2B). It appears that further increases in intracellular ^{64}Cu concentration had no effect on MNK relocalization to the PM (Figure 2B).

In order to determine whether copper induced trafficking of MNK to the PM, we compared the rates of such trafficking under elevated copper concentrations, namely 100 and 500 μM copper. While the steady-state levels of MNK at the PM were almost identical after 3 h of exposure to 100 or 500 μM copper, the respective values for $t_{1/2}$ of 31 and 14 min were substantially different, suggesting that copper did induce trafficking of MNK to the PM (Figure 3). Importantly, the accumulation of 0.1 pmol of ^{64}Cu/μg of protein was also observed after 31 and 14 min of treatment of cells with 100 or 500 μM copper (^{64}Cu), respectively (Figure 3, inset), suggesting a threshold copper level. Consistent with these findings, the initial rates of appearance of MNK at the PM were estimated as 0.06 min^{-1} for 100 μM copper and 0.22 min^{-1} for 500 μM copper.

Trafficking of a distinct pool of MNK to the PM

Cell-surface biotinylation allowed us to estimate the maximum level of MNK at the PM of CUR3 cells under copper loading conditions. MNK was biotinylated at the PM following a 3 h incubation of cells in 500 μM copper, and was subsequently precipitated from the total cell lysate using streptavidin–agarose. The level of residual (non-biotinylated) MNK was compared with the level of total cellular MNK. We found that the maximum achievable level of MNK at the PM was $20 \pm 5\%$ of the total cellular MNK. Under basal copper concentrations (0.8 μM Cu)

the cells were cultured in 500 μM copper for 180 min and then returned to the basal medium for 2–60 min and fixed and processed as described in the Experimental section. Each image is presented as an *xy* section (top panel) and an *xz* section (bottom panel) of cells.
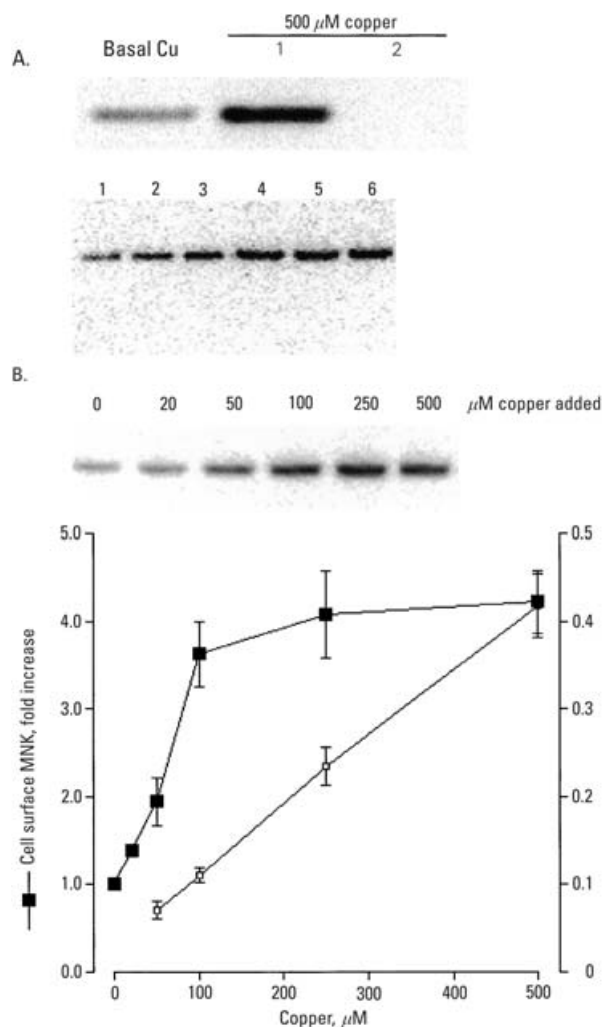
Figure 2   Copper-dependent relocalization of the MNK protein to the PM of CUR3 cells and its cell-surface biotinylation

(**A**) Top panel: CUR3 cells were incubated in basal medium (0.8 μM copper) or in the presence of 500 μM copper for 3 h prior to biotinylation. Biotinylated proteins were precipitated using streptavidin–agarose beads and separated by SDS/PAGE as described in the Experimental section. Biotinylated MNK was detected by Western immunoblotting using anti-MNK antibodies and quantified using phosphorimager and ImageQuant software (see the Experimental section). Shown is a typical Western immunoblot of streptavidin–agarose-precipitated biotinylated MNK from CUR3 cells, demonstrating the level of MNK at the PM in the absence ('Basal Cu') or presence of 500 μM copper. Lanes 1 and 2 show the results of two sequential precipitations with streptavidin-bound agarose (see the Experimental section). Bottom panel: Western immunoblot of MNK, where lanes 1–3 had cell-surface biotinylated MNK from CUR3 cells exposed to 0, 20 and 50 μM added copper, respectively. Lanes 4–6 show MNK in the total cell lysate of CUR3 cells used in lanes 1–3. (**B**) CUR3 cells were exposed to various copper concentrations for 3 h prior to biotinylation (see the Experimental section). Shown is a representative Western blot of biotinylated MNK at the cell surface. The graph represents the mean $\pm$ S.E.M. ($n = 3$) of fold increase of MNK at the PM (■). Values were standardized to samples where no copper was added to demonstrate an increase of MNK at the PM. Also shown (□) are intracellular levels of ^{64}Cu following 3 h incubations at 37 °C in medium supplemented with varying ^{64}Cu concentrations (see the Experimental section).

there was at least a 4–5-fold lower level of MNK at the PM (see above; Figures 2 and 3). To investigate whether this observation was a result of saturation of MNK-retention sites at the PM, the same experiment was performed using CUR2 cells, which express an approx. 5-fold lower level of MNK than CUR3 cells [13]. The results were consistent with those described above: CUR2 cells maintained a steady-state level of MNK under elevated copper concentrations, with 21.5 ± 4.7 % of total cellular pool of MNK
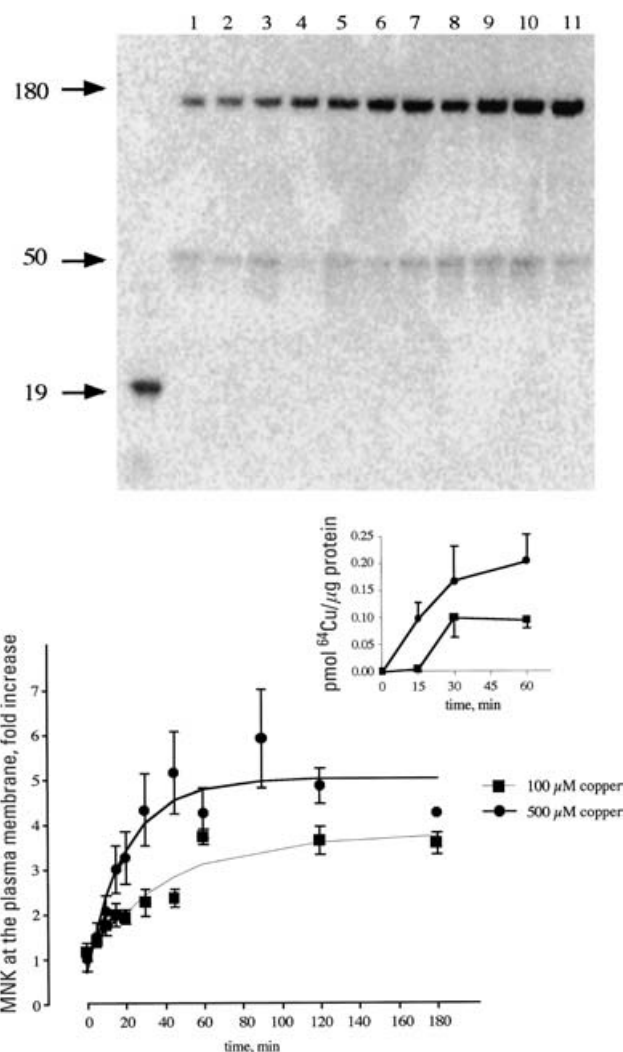

Figure 3   Time-dependent relocalization of MNK to the PM

The cell-surface MNK was biotinylated following exposure of CUR3 cells to 100 μM (■) or 500 μM copper (●) for 5–180 min (see the Experimental section). Biotinylated MNK was detected by Western immunoblot analysis and quantified as described in the Experimental section. The top panel shows a typical Western immunoblot of biotinylated MNK from CUR3 cells treated with 500 μM copper for 0, 5, 10, 15, 20, 30, 45, 60, 90, 120 and 180 min, corresponding to lanes 1–11. A clear and distinct biotinylated MNK band (approx. 180 kDa) is present, as well as a faint lower-molecular-mass non-specific band (approx. 50 kDa), which is also observed in CHO cells not overexpressing MNK (results not shown). A 19 kDa band detected by the secondary antibody corresponds to myoglobin in the molecular-mass standard. On the bottom panel values were standardized to samples treated with 500 μM copper for 180 min and the data are expressed as mean $\pm$ S.E.M. ($n = 4$). Curve fitting was according to the Experimental section (correlation coefficient for 100 μM copper, $r = 0.95$, and for 500 μM copper, $r = 0.95$). The inset represents intracellular ^{64}Cu accumulation following the treatment of cells with 100 or 500 μM ^{64}Cu (symbol designation as above).

seen at the PM, despite much lower overall MNK levels. Furthermore, unlike in the case of CUR3 cells (Figure 1; [4]), an insignificant level of MNK was observed at the TGN of CUR2 cells exposed to elevated copper concentrations ([4]; results not shown). We reported previously that under these experimental conditions MNK trafficking was independent of *de novo* protein synthesis or degradation [4]. We have observed a high stability of MNK, with a $t_{1/2} > 48$ h (R. Fernando, I. Voskoboinik and J. Camakaris, unpublished work).

To determine whether all or only a specific pool of MNK can traffic to the PM in the presence of copper, we biotinylated the
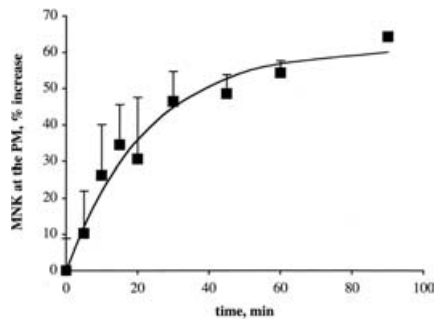
Figure 4   Time course of MNK return to the cell surface from the TGN

The cells were exposed to 500 μM copper for 3 h, biotinylated, and returned to medium with no added copper for 2 h to allow endocytosis of MNK. Medium containing 500 μM copper was once again added to the cells for 5–90 min, and at various time points the cell-surface biotin was cleaved (see the Experimental section). The vertical axis shows the percentage of biotinylated MNK that returns to the PM expressed as mean $\pm$ S.E.M. ($n = 4$). Curve fitting was according to the Experimental section (correlation coefficient, $r = 0.98$).

cell-surface MNK following a 3 h exposure of cells to 500 μM copper, and then allowed the protein to undergo endocytosis, presumably to the TGN (Figure 1; [4]), for 2 h. By subjecting the cells once again to 500 μM copper for the indicated times and then cleaving the surface biotin with GSH, we measured the amount of internalized biotinylated MNK and estimated the rate of return of biotinylated MNK to the cell surface. If this was a random process, we would expect a steady-state return of approx. 20 % (see above) of the labelled MNK to the PM. However, at the steady-state conditions in elevated copper, approx. 55 % of the originally biotinylated MNK appeared to return to the cell surface (Figure 4). Figure 4 also indicates that MNK returning to the PM reaches a steady-state level with a $t_{1/2}$ of 17 min, a value similar to that presented in Figure 3 ($t_{1/2}$ of 14 min at 500 μM Cu). These results indicate that trafficking of MNK to the PM is not a random process, and suggest the presence of a specific copper-responsive pool of the protein.

The effect of copper on MNK endocytosis

To determine whether copper influences the rate of MNK endocytosis (or internalization), we used a reversible biotinylation technique. As depicted in Figure 5, rapid endocytosis of MNK from the PM occurs both in the absence of added copper and in the presence of 500 μM copper, with the retention of MNK at the PM being estimated as $t_{1/2}$ values of 5 and 4 min respectively. However, the steady-state levels of internalized MNK under these conditions were significantly different. Thus in the absence of copper only 25 % of the originally labelled MNK was found at the PM under steady-state conditions (Figure 5), consistent with the 4–5-fold steady-state increase in the level of MNK at the PM found in the above experiments (Figures 2 and 3). In contrast, in the presence of elevated copper approx. 50 % of the originally labelled MNK was found at the PM (Figure 5).

These observations suggest either that a proportion of MNK was retained at the PM or that the internalized MNK rapidly recycled back to the PM. To resolve this dilemma, we utilized a procedure involving double cleavage of biotinylated MNK. Thus MNK trafficking to the PM was stimulated by 500 μM copper for 3 h. Cell-surface MNK was then biotinylated and allowed to internalize for 15 min in the presence of 500 μM copper to reach the apparent steady-state level of internalization (Figure 5B). The cell-surface biotin was subsequently cleaved with GSH, and the cells were incubated in 500 μM copper once again for indicated times, 1–
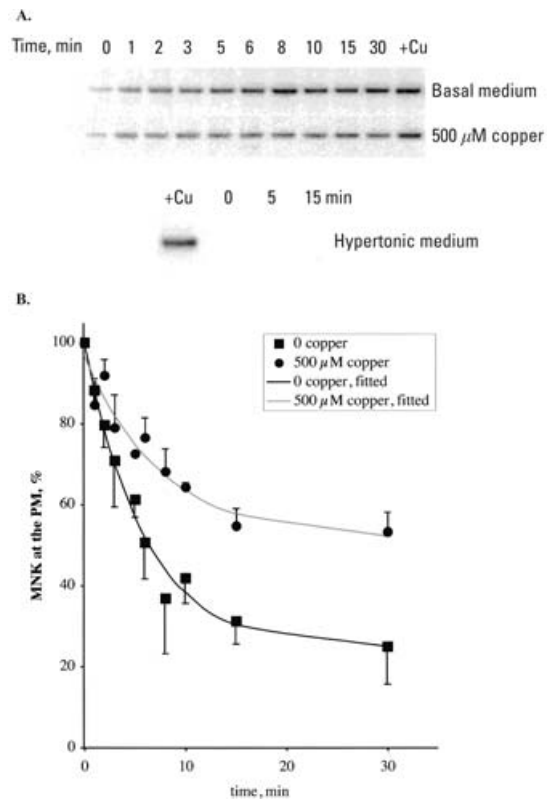

Figure 5   Endocytosis of MNK in the absence or presence of elevated copper

CUR3 cells were exposed to medium containing 500 μM copper for 3 h prior to biotinylation. Cells were then incubated in the absence (■) or presence (●) of 500 μM copper. At various time points, 0–30 min, the cell-surface biotin was cleaved (see the Experimental section). (**A**) Shown are representative Western immunoblots of MNK precipitated using streptavidin–agarose. In a control experiment, following copper treatment, the cells were exposed to hypertonic medium in order to demonstrate that blocking endocytosis would result in a complete cleavage of biotinylated MNK. (**B**) Quantitative analysis. Data are expressed as mean $\pm$ S.E.M. ($n = 3$) of percentage of biotinylated MNK at the PM compared with time 0 (which corresponds to a 3 h incubation in 500 μM copper). The values are derived from the proportion of internalized MNK protected from cleavage by glutathione at the cell surface. Curve fitting was according to the Experimental section (correlation coefficient for 500 μM copper, $r = 0.97$, and for basal medium, $r = 0.99$).

15 min, to allow trafficking to the PM of internalized biotinylated MNK (Figure 6). By subjecting the cells to a second round of cleavage with GSH, we could estimate the proportion of biotinylated MNK that has recycled back to the PM. Figure 6 indicates that the labelled pool of MNK has undergone rapid recycling to the PM with a $t_{1/2}$ of 1 min and the rate estimated as 3.5 min^{-1}. These parameters indicated the presence of a fast-recycling pool of MNK in the presence of added copper. Furthermore, under steady-state conditions 50 % of biotinylated MNK was found at the PM, while the remaining half appeared to be internalized (Figure 6). These findings provide further evidence that copper does not inhibit MNK internalization from the PM, but rather facilitates its entry (or retention) into a fast-recycling pool.

DISCUSSION

In this study we aimed to investigate whether addition of copper results in the steady-state relocalization of MNK from the TGN to the PM by influencing its trafficking to the PM and/or endocytosis from the PM. Our findings provide the first evidence for a dual effect of elevated copper levels on MNK trafficking: facilitation
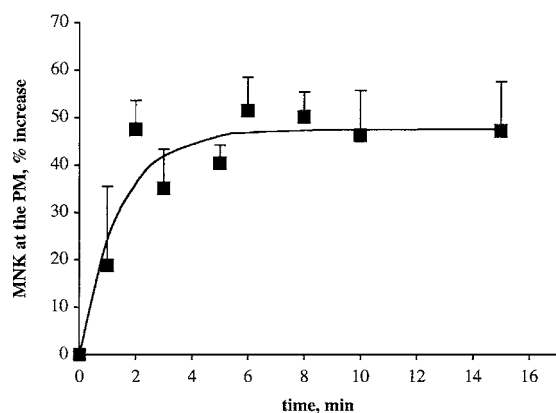
Figure 6   MNK recycling in elevated copper

Cell-surface MNK was biotinylated following exposure of cells to 500 μM copper for 3 h. The cells were returned to 500 μM copper medium for 15 min, followed by cleavage of biotin at the cell surface (see the Experimental section). Subsequently, the cells were returned to 500 μM copper medium for 1–15 min to allow recycling of internalized biotinylated MNK back to the PM. At various time points the cell-surface biotin was cleaved for a second time. The graph shows the percentage of internalized biotinylated MNK that reappears at the PM. Values are adjusted to time 0, and are expressed as the mean $\pm$ S.E.M. ($n = 3$). Curve fitting was according to the Experimental section (correlation coefficient, $r = 0.94$).

of recruitment of MNK into the trafficking pathway to the PM and, subsequently, diversion into a fast-recycling pool.

Earlier and current studies on MNK trafficking have indicated that the protein can recycle constitutively between the TGN and the PM in basal extracellular copper concentrations (0.8 μM copper), and can undergo a copper-stimulated steady-state relocalization to the PM (Figure 1; [4,6]). However, it was not known whether all intracellular MNK responds to copper by trafficking to the PM or if there are distinct (e.g. segregated) copper-responsive pool(s) of the protein. We have employed biotin labelling of cell-surface proteins to attempt to answer these questions. We determined that the proportion of MNK localized to the PM under steady-state elevated copper conditions (500 μM copper) was approx. 20 % of the total MNK, regardless of the total amount of MNK present in the cell. Furthermore, in these studies we found that the MNK protein, which was labelled at the PM, could be internalized, presumably to the TGN (Figure 1; [4]), upon the removal of elevated copper from the culture medium (Figures 4 and 5). When these cells are returned to elevated copper medium, trafficking to the PM will be triggered. If MNK were to be randomly recruited into the trafficking pathway to the PM, we would expect only 20 % of the originally labelled (at the cell surface) MNK to reappear at the PM. However, approx. half of the biotinylated MNK reappeared at the PM under elevated copper concentrations (Figure 4). A plausible explanation for this result is the existence of a defined copper-responsive pool of MNK, which is capable of trafficking to the PM in response to copper. Further experiments indicated that in the presence of elevated copper, under steady-state conditions, the copper-responsive pool of MNK apparently consists of approximately equal amounts of PM-localized and internalized molecules of MNK (Figure 6). Taken together, our data suggest that approx. 40 % of total MNK responded to elevated copper by relocalizing to the PM, where 20 % was detected at the PM and an approximately equal amount, 20 %, was internalized as a part of the fast-recycling pool (Figure 6). Presumably the remaining MNK performs other intracellular biochemical functions, such as the delivery of copper to cuproenzymes in the secretory pathway. At the PM, MNK functions in copper efflux [1,4].

The analysis of MNK internalization from the PM in the presence or absence of added extracellular copper indicated almost identical $t_{1/2}$ values, 4 and 5 min respectively (Figure 5), suggesting that copper did not exert a significant effect on MNK endocytosis from the PM. Furthermore, observed differences in the steady-state level of MNK internalization did not appear to be due to the retention of MNK at the PM, but were rather due to its rapid recycling in the presence of elevated copper to the PM through the fast-recycling pool (Figure 6). It is important to note that we have shown in the past that, under similar experimental conditions, a labile pool consisting of at least 60 % of accumulated ^{64}Cu was effluxed from CUR3 cells within 30 s after returning the cells to basal medium [13]. The remaining ^{64}Cu was effluxed over a much longer period of time, most likely because of mobilization of ^{64}Cu into a slow-exchangeable pool of copper, and did not correlate with the level of MNK in the cells [13]. Therefore, the removal of elevated copper from the medium results in a very rapid loss of a labile pool of copper, which we believe is responsible for MNK trafficking [13]. We found here that approximately half of the PM MNK was internalized in the presence of elevated copper under steady-state conditions (Figure 5), but rapidly returned to the PM ($t_{1/2}$ of 1 min and an initial rate of 3.5 min^{-1}; Figure 6). In comparison, MNK trafficking from the TGN to the PM in the presence of 500 μM copper occurred with a $t_{1/2}$ of 14 min and an initial rate of 0.22 min^{-1} (Figure 3). This marked, almost 15-fold, difference suggests that the fast-recycling pathway is not likely to involve trafficking through the TGN. Furthermore, this highlights a postulated role for copper in diverting MNK into a fast-recycling pool, or in inhibiting MNK entering the endocytic pathway to the TGN. The latter could occur via endosomal compartment(s) [16], as has been reported for other systems, e.g. GLUT4, TGN38 and furin [17,18].

Recent studies [16] have utilized PM lawns to demonstrate a slow rate of copper-dependent trafficking of MNK to the PM with a $t_{1/2}$ of 60 min. A possible reason for such differences may lie in variation in kinetics of copper uptake, accumulation and distribution in HeLa cells used in those studies [16] and the CHO cells used here (e.g. influences of metallothionein levels on copper distribution).

Does copper stimulate trafficking of MNK to the PM? Due to the low levels of MNK at the PM in basal medium, we were unable to estimate the $t_{1/2}$ under these conditions. Instead we investigated the kinetics of MNK appearance at the PM at two different concentrations of added copper, namely 100 and 500 μM. Whereas similar steady-state levels of MNK at the PM were observed following a 3 h exposure of cells to these concentrations of copper, the $t_{1/2}$ values were estimated as 31 and 14 min at 100 and 500 μM added copper, respectively (Figure 3). These data suggest that in basal copper medium (0.8 μM copper) the constitutive trafficking of MNK to the PM is likely to be even slower than a $t_{1/2}$ of 31 min, which would be consistent with slow constitutive recycling of MNK observed by immunofluorescence microscopy [6], and also with slow constitutive trafficking of the TGN resident proteins to the PM. For example, furin and TGN38 have been shown to have a $t_{1/2}$ value of 35 and 45 min, respectively [18]. Faster rates of relocalization of MNK to the PM at higher copper concentrations coincided with higher levels of intracellular copper accumulation in 500 μM extracellular copper compared with 100 μM copper (Figures 2 and 3). It appears that once a presumptive threshold intracellular copper concentration was reached (corresponding to an accumulation of 0.1 pmol of ^{64}Cu/μg of protein), the amounts of MNK at the PM were similar irrespective of the concentration of extracellular copper added (Figures 2 and 3). Given our evidence for a fast-recycling pool of MNK, it is possible that the steady-state relocalization of MNK to the PM may,

at least partly, be attributed to copper-concentration-dependent recruitment/retention of MNK in that pool.

An endocytic compartment, which is involved in the recycling of a glucose transporter, GLUT4, whose trafficking is regulated by insulin, has been described previously [17]. In this postulated system GLUT4 formed a recycling pool utilizing sorting and/or recycling endosomes as well as uncharacterized trafficking pools, with $t_{1/2}$ values being similar to those reported in our current study. Previously we provided evidence that MNK utilizes a similar pathway for internalization from the PM and the return to the TGN [9] (Figure 5A). Our recent studies indicated a possible link between a specific catalytic conformation of MNK and its trafficking and retention at the PM [11,12]. MNK is postulated to undergo conformational changes, as part of the catalytic cycle, and these may influence its translocation from the TGN to the PM and subsequent 'entrapment' within the fast-recycling pool [11,12].

In conclusion, the studies reported here provide the first detailed insight into the role of copper on the dynamics and mechanisms of trafficking of the Menkes copper translocating P-type ATPase. We propose a dual role for copper in the regulation of MNK relocalization from the TGN to the PM: (i) copper activates relocalization of MNK from the TGN by recruiting MNK into a trafficking pathway to the PM and (ii) copper stimulates recruitment/retention of MNK in a fast-recycling pool, presumed to be proximal to the PM. Furthermore our data suggest that there are likely to be at least two pools of MNK in the cell, with one of these responding to copper by vesicular trafficking to the PM. We believe that this segregation of MNK in different trafficking pools is a major factor responsible for the dual role of MNK in copper homoeostasis, the delivery of copper to cuproenzymes in the secretory pathway and the efflux of excess copper from the cell.

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