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Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking

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The Menkes P-type ATPase (MNK), encoded by the Menkes gene (*MNK; ATP7A*), is a transmembrane copper-translocating pump which is defective in the human disorder of copper metabolism, Menkes disease. Recent evidence that the MNK P-type ATPase has a role in copper efflux has come from studies using copper-resistant variants of cultured Chinese hamster ovary (CHO) cells. These variants have *MNK* gene amplification and consequently overexpress MNK, the extents of which correlate with the degree of elevated copper efflux. Here, we report on the localization of MNK in these copper-resistant CHO cells when cultured in different levels of copper. Immunofluorescence studies demonstrated that MNK is predominantly localized to the Golgi apparatus of cells in basal medium. In elevated copper conditions there was a rapid trafficking of MNK from the Golgi to the plasma membrane. This shift in steady-state distribution of MNK was reversible and not dependent on new protein synthesis. In media containing basal copper, MNK accumulated in cytoplasmic vesicles after treatment of cells with a variety of agents that inhibit endosomal recycling. We suggest that MNK continuously recycles between the Golgi and the plasma membrane and elevated copper shifts the steady-state distribution from the Golgi to the plasma membrane. These data reveal a novel system of regulated protein trafficking which ultimately leads to the efflux of an essential yet potentially toxic ligand, where the ligand itself appears directly and specifically to stimulate the trafficking of its own transporter.

Keywords: copper transport/Golgi/P-type ATPase/trafficking

Introduction

Copper is a trace element with redox properties which make it essential for aerobic cells. However, these same properties can result in toxicity if appropriate copper levels are not maintained. Consequently, intracellular copper levels must be carefully controlled, presumably by regulated transport mechanisms. Disruptions of some of these mechanisms can have a genetic basis as illustrated in the case of both Menkes disease and Wilson disease (Danks, 1995). Menkes disease is caused by mutations in the *MNK* (*ATP7A*) gene (Chelly *et al.*, 1993; Mercer *et al.*, 1993; Vulpe *et al.*, 1993), while the gene defective in Wilson disease is *WND* (*ATP7B*) (Bull *et al.*, 1993; Tanzi *et al.*, 1993; Yamagachi *et al.*, 1993). These genes are closely related and encode proteins which are members of the P-type ATPase family. P-type ATPases are involved in the ATP-dependent transport of cations across membranes in both prokaryotes and eukaryotes (Pederson and Carafoli, 1987). There are several examples of copper-transporting P-type ATPases from bacteria (Odermatt *et al.*, 1993; Kanamaru *et al.*, 1994), yeast (Rad *et al.*, 1994; Fu *et al.*, 1995) and mammals (Bull *et al.*, 1993; Chelly *et al.*, 1993; Mercer *et al.*, 1993; Tanzi *et al.*, 1993; Vulpe *et al.*, 1993; Yamagachi *et al.*, 1993), each with one or more putative copper-binding motifs (minimally GMXCXXC) close to the amino-terminus.

As cultured fibroblasts from Menkes patients accumulate copper (Goka *et al.*, 1976; Horn, 1976; Camakaris *et al.*, 1980), it was suggested that the Menkes P-type ATPase (MNK) may be involved in the efflux of copper (Camakaris *et al.*, 1982). This hypothesis has recently been strongly supported by studies on copper-resistant CHO cells in which *MNK* gene amplification (up to 70-fold) has resulted in elevation of *MNK* mRNA and protein levels, and a corresponding increase in copper efflux (Camakaris *et al.*, 1995). This study focuses on the regulation of the *MNK* gene by copper and the subcellular localization of MNK in the copper-resistant CHO cells which overexpress MNK. The results demonstrate that the expression of the *MNK* gene homologue in CHO cells is not regulated by copper at the level of transcription or translation, but rather that copper influences the subcellular localization of the protein. In cells grown in medium without added copper, the MNK protein was localized to the Golgi apparatus. However, when media copper levels were elevated, MNK was trafficked to the plasma membrane from where copper is extruded from the cell. When cells were returned to basal medium, MNK was rapidly recycled back to the Golgi region. Both the endocytic and exocytic trafficking of MNK were ATP-dependent and did not require new protein synthesis. These results establish the existence of a novel ligand-inducible system of regulated protein trafficking.

Results

Effect of copper on the subcellular localization of MNK

In previous work, the copper-resistant CHO cell lines, CUR1, CUR2 and CUR3, were isolated as spontaneous variants of the parental CHO-K1 cell line using a stepwise copper-selection protocol (Camakaris *et al.*, 1995). CUR1,

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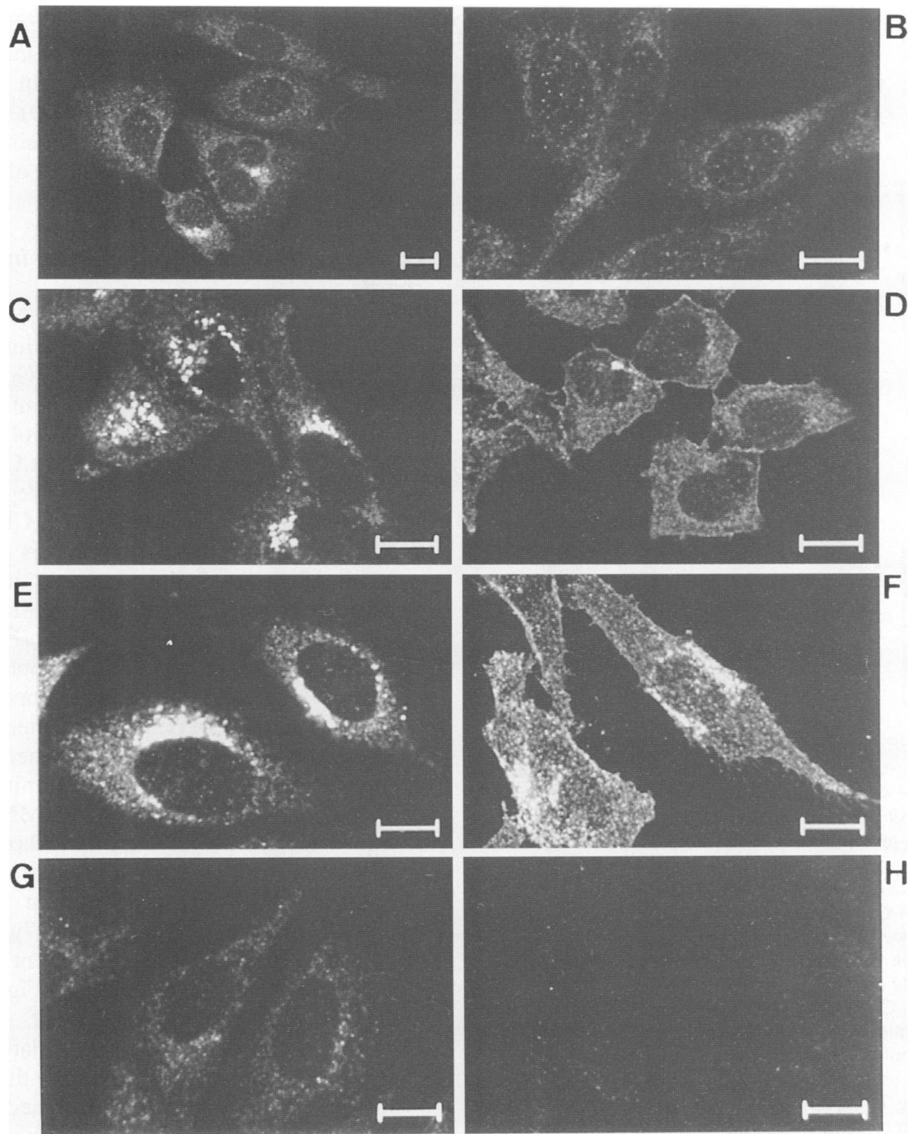

Fig. 1. Effect of copper on intracellular localization of MNK in CHO cell lines. The copper-resistant variant cells, CUR1, CUR2 and CUR3, grown for 48 h in basal media (**A**, **C** and **E**, respectively) or media supplemented with 189 μM copper ($CuCl_2$) (**B**, **D** and **F**, respectively), were stained by indirect immunofluorescence with antibodies to MNK (1:50). As controls, CUR3 cells grown in basal medium were treated with pre-immune serum (1:50) (**H**) or with the MNK antibodies which had been preabsorbed with the MNK-GST fusion protein used for immunization (**G**). After primary antibody reactions, cells were reacted with FITC-conjugated sheep antibodies to rabbit IgG (1:200). Bars, 10 μm. Quantitation of fluorescence within the perinuclear region of CUR2 cells grown in basal medium revealed an average pixel intensity (± SD) of 36.0 ± 6.6. The average pixel intensity decreased to 16.1 ± 5.0 in the perinuclear region from cells in high copper. This difference was significant ($P < 0.001$, Student's *t*-test).

CUR2 and CUR3 have 2-, 10- and 70- fold amplifications of the *MNK* gene, respectively, and the levels of MNK protein are similarly elevated (Camakaris *et al.*, 1995). As the increase in MNK protein in these CHO variants paralleled the enhanced biological activity of MNK [copper efflux (Camakaris *et al.*, 1995)], these variants are ideal for studies of subcellular localization of the protein. Using confocal immunofluorescence microscopy with antibodies raised against the amino-terminal 590 amino acids of MNK, the localization of the MNK protein was investigated in each cell line cultured in basal medium (0.8 μM Cu). Fluorescent staining was observed at the perinuclear region in CUR1, CUR2 and CUR3 (Figure 1A, C and E), suggesting MNK localization in the Golgi apparatus. The intensity of this perinuclear signal in each of the CUR cell lines paralleled the amount of MNK protein previously detected using Western blots (Camakaris *et al.*, 1995). In the MNK-overexpressing cell line, CUR3, the perinuclear fluorescence was decreased significantly when the MNK antibodies were preincubated with the MNK–glutathione-*S*-transferase (GST) fusion protein (Camakaris *et al.*, 1995) used for immunization (Figure 1G), and no staining was observed with preimmune serum (Figure 1H).

When cells were cultured for 24 h in the presence of 189 μM copper, MNK was redistributed from the perinuclear region and showed a dispersed punctate staining pattern in the cytoplasm of CUR1, CUR2 and CUR3 (Figure 1B, D and F), suggesting localization of MNK in vesicles. Comparison of average pixel intensities of digitized confocal images showed there was a 2.3-fold decrease in the perinuclear region of CUR2 cells in elevated copper relative to cells in basal medium. In CUR2 and CUR3 cells (Figure 1D and F), which have

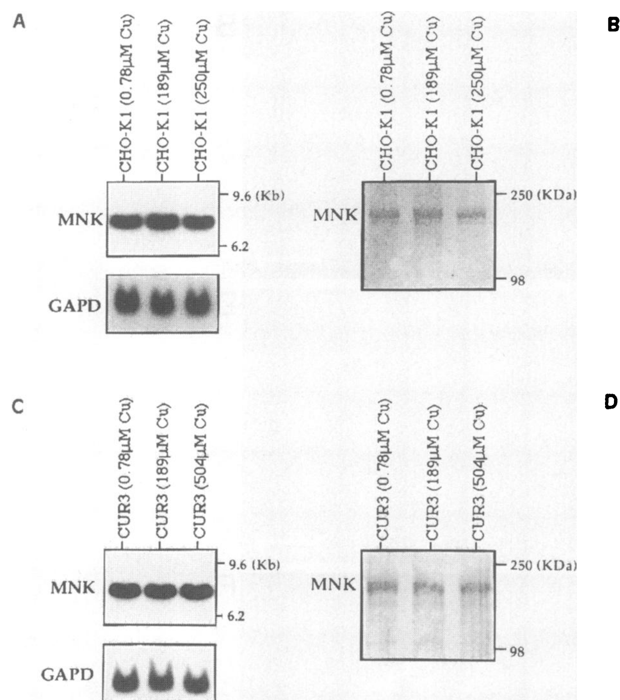

Fig. 2. Analysis of the effect of copper on *MNK* mRNA and MNK protein levels. Northern blot (**A**) and Western blot (**B**) from parental CHO-K1 cells grown for 48 h in medium containing 0.78 µM Cu (basal media copper), 189 µM Cu and 250 µM Cu, showing levels of *MNK* mRNA and MNK protein, respectively. Northern blot (**C**) and Western blot (**D**) showing levels of *MNK* mRNA and protein in CUR3 cells grown for 48 h in basal medium, and medium containing 189 µM Cu and 504 µM Cu. Northern blots containing 10 µg total RNA per track were probed with a hamster *MNK* cDNA clone-spanning regions between base pairs 2331–4041 (Camakaris *et al.*, 1995). Due to the 70-fold increase in *MNK* mRNA in CUR3, RNA blots from CHO-K1 and CUR3 cells were exposed at –70°C for 72 h and 3 h, respectively. Equivalence in RNA loadings was established using both ethidium bromide staining and by probing the same filters with a glyceraldehyde-3-phosphate dehydrogenase probe (*GAPD*). Western blots, containing 25 µg total protein per track, were probed with the MNK antiserum (1:300). Due to differences in MNK protein levels, the development time for protein detection was 5 min for the Western blot with CHO-K1 and 30 s for the Western blot with CUR3 protein.

the highest levels of MNK, there was strong fluorescence outlining the cell periphery, suggesting significant levels of MNK associated with the plasma membrane. In the parental cell line, CHO-K1, there was a weak perinuclear signal with some cytoplasmic dispersion in basal medium and there was no apparent change in elevated copper (data not shown). The CHO cell lines showed no significant drop in cell viability under the copper load conditions described above (data not shown).

The amount of MNK mRNA and protein is not affected by media copper concentration

Northern and Western blots were used to determine if the copper-induced relocalization of MNK was accompanied by changes in the levels or size of either *MNK* mRNA or MNK protein. As shown in Figure 2A and C, the amounts of *MNK* mRNA in CHO-K1 and CUR3 cells were not significantly changed by growth in a high-copper medium. This is consistent with the previous conclusions of Paynter *et al.* (1994) that transcription of the *MNK* gene in mice is unlikely to be regulated by copper. Similarly, Western blots of extracts from cells grown in low- and high-copper media did not indicate any change in the amount or size of MNK protein (Figure 2B and D). Thus, the copper-induced relocalization of the protein does not appear to involve alterations in amounts of *MNK* mRNA or MNK protein.

Localization of the MNK protein in low-copper media

The perinuclear localization of MNK in the copper-resistant cells cultured in basal medium (Figure 1) suggested MNK localization in the Golgi apparatus. To investigate this further, the distribution of MNK was compared with that of the *trans*-Golgi network (TGN) protein, p230 (Kooy *et al.*, 1992; P.A.Gleeson and G.Griffiths, unpublished data). Extensive regions of overlap of MNK with p230 were observed in CUR3 cells cultured in basal medium (Figure 3A–C). This suggests that MNK is localized to the Golgi apparatus in cells cultured in basal medium. Significantly, the localization of p230 was unaffected by elevated copper levels (Figure 3E), whereas in the same cell MNK was redistributed (Figure 3D and F). Hence, while MNK and p230 appear to reside in the same organelle, the effect of elevated copper levels on localization is specific to MNK. When CUR3 cells were cultured in the presence of the antimitotic drug, nocodazole, the perinuclear localization of MNK was completely disrupted (Figure 4A). Since nocodazole depolymerizes microtubules and causes disruption of the Golgi apparatus (Rogalski and Singer, 1984; Tassin *et al.*, 1985), this dispersion of MNK was consistent with localization in the Golgi apparatus and is consistent with the apparent co-localization of MNK and p230 (Figure 3C).

The Golgi apparatus consists of stacks of cisternal elements connected to a reticular lattice known as the TGN. In order to more closely define the location of MNK within the Golgi, cells were treated with the fungal metabolite, brefeldin A (BFA), which affects the morphology of the Golgi cisternae and the TGN in two very different ways. The Golgi cisternae are resorbed into the endoplasmic reticulum in cells treated with BFA (Lippincott-Schwartz *et al.*, 1990). BFA induces the TGN initially to fuse with portions of the endosomal pathway (Wood *et al.*, 1991), and with longer exposures to condense to a juxtanuclear structure known as the microtubule-organizing centre (Ladinsky and Howell, 1992). Hence, BFA is very useful in identifying whether a Golgi protein resides in the cisternae or the TGN. Treatment of CUR3 cells with BFA resulted in resorption of the *cis-/medial-*Golgi membrane protein, mannosidase II, into the endoplasmic reticulum (Figure 4G compared with 4F), an expected effect of BFA on proteins in the Golgi cisternae (Lippincott-Schwartz *et al.*, 1990). The TGN marker protein, TGN38, contracted to a tight juxtanuclear location in cells treated with BFA (Figure 4E, compare with 4D), as demonstrated previously (Ladinsky and Howell, 1992; Reaves *et al.*, 1992; Molloy *et al.*, 1994). The effect of BFA on the localization of the MNK protein was similar to that for TGN38, with contraction of MNK to a juxtanuclear location (Figure 4C, compare with 4B). Hence, MNK is likely to be localized in the TGN, rather than the Golgi stack.

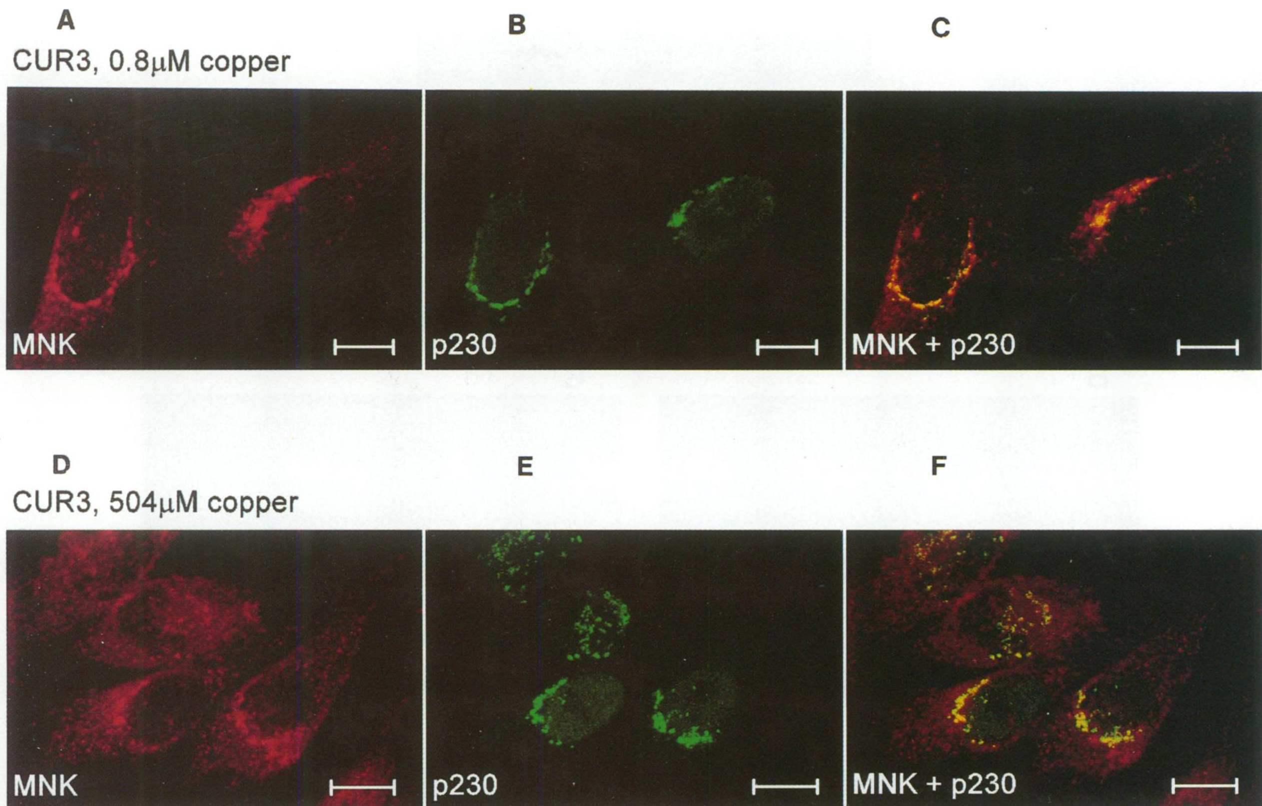

Fig. 3. Comparison of the subcellular distributions of MNK with the TGN protein p230 using double labelling. (**A**) CUR3 cells grown in basal medium were stained using MNK antiserum followed by Texas red-conjugated donkey anti-rabbit IgG (shown in red). (**B**) The same cells as in (A) were incubated with affinity-purified human antibodies to p230 followed by FITC-conjugated sheep antibodies to human Ig (shown in green). (**C**) Superimposed images of (A) and (B) reveals regions of MNK and p230 co-localization (shown in yellow). The locations of MNK (**D**) and p230 (**E**) in CUR3 cells cultured in 504 μM copper are indicated with merged images showing regions of co-localization (**F**). Bars, 10 μm.

Elevated copper levels result in an increase in MNK associated with the plasma membrane

The fluorescent staining at the peripheries of CUR2 and CUR3 cells cultured in the presence of elevated copper levels (Figure 1D and F) suggested a net increase in MNK associated with the plasma membrane. To examine this further we analysed the level of MNK in highly purified plasma membrane (PM) lawns. Procedures for obtaining these PM lawns involve sonication of cells grown on coverslips to yield PM fragments with their cytosolic surfaces exposed and which are free from contamination by intracellular membranes (Moore *et al.*, 1987; Heuser, 1989; Lin *et al.*, 1991). Plasma membrane lawns isolated by this technique retain structural properties such as caveolae, clathrin-coated pits and cytoskeletal structures (Lin *et al.*, 1991; Robinson *et al.*, 1992). Immunofluorescence labelling of PM lawns by laser scanning confocal microscopy has been used as an assay to quantitate the movement of the glucose transporter, GLUT4, from the TGN and associated tubulovesicular structures to the plasma membrane in response to insulin (Piper *et al.*, 1992, 1993; Robinson *et al.*, 1992). This procedure was used to the investigate whether, in the presence of elevated copper levels, there is a net increase in the levels of MNK associated with plasma membrane. In basal medium, the levels of MNK associated with the plasma membrane of CUR3 cells were low (Figure 5A). However, in cells cultured in elevated copper, there was a striking increase in MNK associated with the plasma membrane (Figure 5B). On the basis of average pixel intensity of digitized confocal microscope images, there was a 5.5-fold increase in MNK levels associated with the plasma membrane lawns derived from cells grown in high-copper medium relative to cells cultured in basal medium. Controls indicated that the membranes attached to the coverslips were derived from the plasma membrane, as shown by strong fluorescence observed using antibodies to a marker for the plasma membrane, Na^+/K^+ ATPase (Figure 5C). No difference in labelling of PM fragments from cells grown in elevated copper relative to basal medium was observed using antibodies to Na^+/K^+ ATPase (data not shown).

Immunoelectron microscopy was performed for CUR3 cells cultured initially in basal medium and then in elevated copper medium. Pre-embedding labelling with MNK antibodies of CUR3 cells cultured in basal medium resulted in gold particles on clusters of small vesicles of ~60 nm in diameter (Figure 6A). No particles were associated with other cytoplasmic membranes, or the plasma membrane (lytic fixation permitted access of reagents to the cytoplasm but did not preserve Golgi structure). Significantly, gold particles were observed on the plasma membrane only when CUR3 cells were cultured in elevated copper, and this labelling was always associated with the cytoplasmic side of the membrane (Figure 6B and C). Vesicle labelling was also observed in high-copper medium (Figure 6B and C). Taken together with the immunofluorescence data, these observations support the hypothesis that elevation of intracellular copper levels results in relocalization of MNK to the plasma membrane.

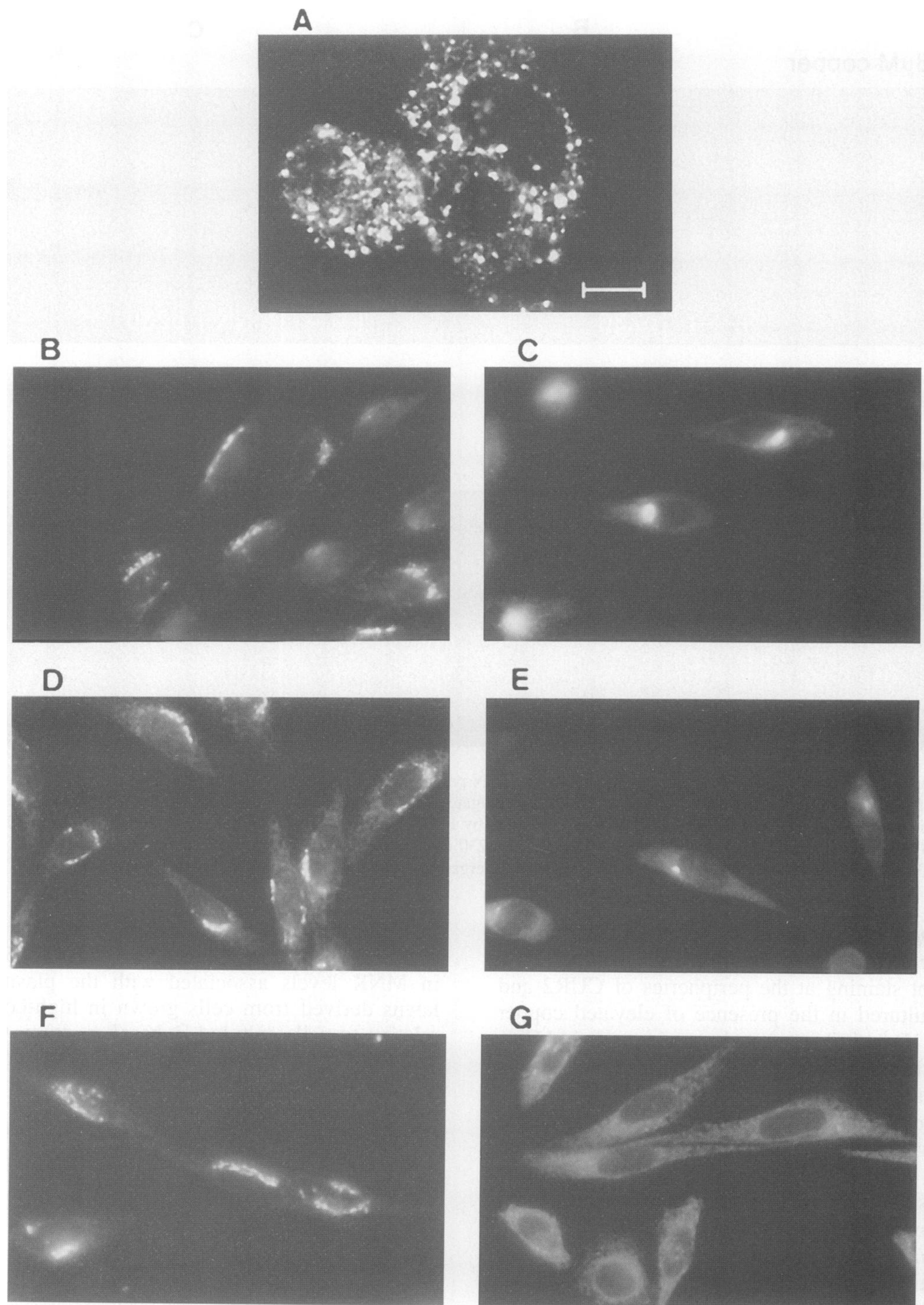

Fig. 4. Effects of nocodazole and brefeldin A on the intracellular distribution of MNK. CUR3 cells were grown for 48 h in basal medium and then incubated with 10 μM nocodazole (**A**), 5 μg/ml brefeldin A (**C**, **E** and **G**), or in the absence of drugs (**B**, **D** and **F**) for 1 h at 37°C. Cells were fixed and then stained using either affinity-purified MNK antibodies (A–C), anti-TGN38 antiserum (D and E) or anti-mannosidase II antiserum (F and G) followed by FITC-conjugated sheep antibodies to rabbit IgG. Bar, 10 μm.

Time-course of the copper-induced relocalization of MNK

To determine the response time for the copper-induced relocalization of MNK, the distribution of MNK was studied at various time intervals after the addition of copper. CUR2 cells were used in these experiments because, both in the presence and absence of elevated copper, the subcellular localization of MNK was most distinct and hence, subtle differences in localization over time were more likely to be apparent. The redistribution of MNK in response to copper was detectable within 10 min of transferring CUR2 cells to the elevated copper medium, where there was a decrease in perinuclear localization as MNK staining became more punctate and diffuse (compare Figure 7A with 7B). This punctate staining was established by 15 min (Figure 7C) and remained so through 1 h (Figure 7D) until 24 h (Figure 7E), with only slight changes over this period. These time intervals

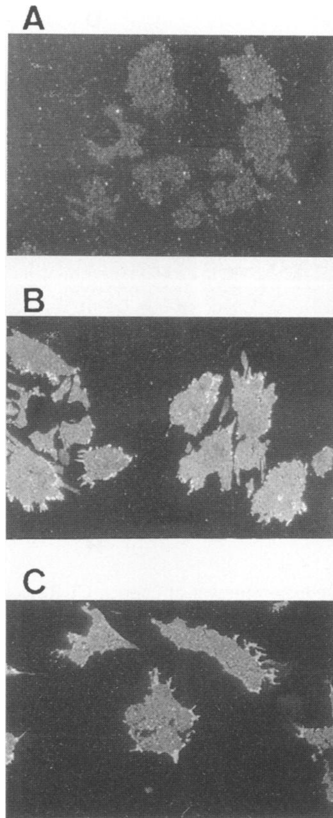

Fig. 5. Localization of MNK in plasma membrane lawns of cells grown in elevated copper. Plasma membrane lawns were isolated and fixed from CUR3 cultured for 48 h in basal medium (**A**) or 189 μM copper (**B** and **C**) before processing for immunofluorescence analysis using either affinity-purified MNK antibodies (A and B) or rabbit antiserum to Na^+/K^+ ATPase (C). Primary antibodies were detected using FITC-conjugated sheep antibodies to rabbit IgG. Quantitation of fluorescence from MNK antibodies, as described in Materials and methods, revealed an average pixel intensity (± SD) of 17.0 ± 3.0 in fragments derived from cells in basal medium. The average pixel intensity increased to 93.3 ± 5.9 in fragments from cells in high-copper media. This difference was significant ($P < 0.001$, Student's *t*-test).

indicate that the copper-induced relocalization of MNK to the plasma membrane was the result of exocytic trafficking rather than *de novo* protein synthesis or differential splicing of *MNK* mRNA. This is supported by the Northern and Western data (Figure 2) and the failure of cycloheximide, which inhibits protein synthesis, to influence this process (Figure 8B, compared with 8A and 8C). Significantly, when cells were transferred back to basal medium, there was reappearance of MNK at the Golgi within 30 min (Figure 7G) and a Golgi staining pattern was fully restored by 1 h (Figure 7H). This is likely to represent recycling of MNK, since the process was not dependent on new protein synthesis, as shown by the lack of effect of cycloheximide (Figure 8E compared with 8D and 8F). The rapid return of MNK to the Golgi region suggests that the relocalization of MNK caused by the elevated copper level was not due to the metal's toxicity.

ATP is required for the copper-regulated trafficking of MNK

To determine whether the copper-regulated trafficking of MNK from the Golgi to the plasma membrane was ATP-

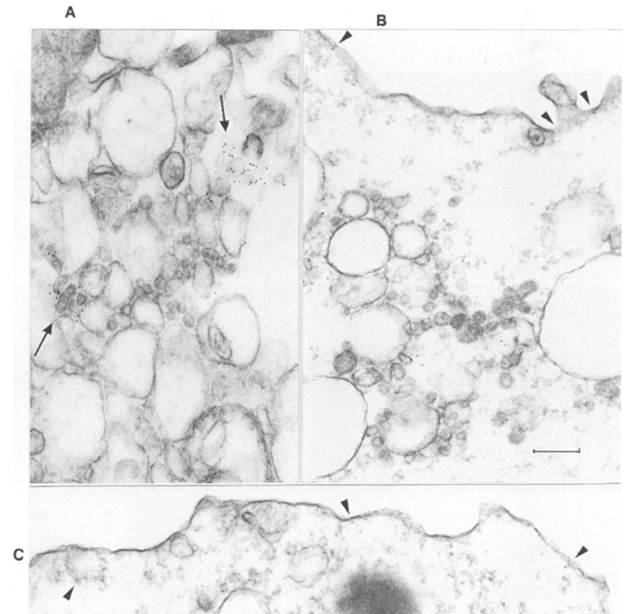

Fig. 6. Immuno-gold localization of MNK in CUR3 cells cultured in basal- and copper-supplemented medium. (**A**) Electron micrograph showing clusters of gold-labelled small vesicles (indicated by arrows) after pre-embedding labelling with the MNK antiserum (1:100) within a disrupted CUR3 cell grown in basal medium. (**B**) Low levels of gold labelling at the plasma membrane of a CUR3 cell grown in medium supplemented with 504 μM copper (indicated by arrows), as well as some cytoplasmic small vesicle labelling. (**C**) Plasma membrane-associated labelling from another region of the same cell shown in (B). Bars, 200 nm.

dependent, cellular ATP levels were depleted by incubating cells in medium containing 2-deoxy-D-glucose and sodium azide (DOG/Az) before the transfer of cells to medium with elevated copper. In ATP-depleted cells, the addition of copper failed to induce redistribution of MNK which remained perinuclear (Figure 9F), while in ATP-non-depleted control cells, copper-induced relocalization of MNK was observed (Figure 9E). ATP-depleted cells remained viable for the duration of the experiment, since normal trafficking of MNK was observed when these cells were incubated for 2 h in the presence of 189 μM copper without DOG/Az (data not shown).

ATP-dependence of the transport of MNK from the plasma membrane to the Golgi was also tested. Starting with CUR3 cells that were grown for 24 h in the presence of elevated copper (Figure 9G), ATP was depleted by incubating the cells for 15 min with DOG/Az before removal of copper by transferring cells to basal medium (also containing DOG/Az). The depletion of cellular ATP levels had an inhibitory effect on the recycling of MNK to the Golgi with the fluorescent staining remaining cytoplasmic and punctate (Figure 9H), compared with the compact perinuclear staining which reappeared in ATP-non-depleted control cells (Figure 9I). A subsequent removal of DOG/Az, by incubating the ATP-depleted cells shown in Figure 9H for 2 h in basal medium, resulted in the return of MNK to the Golgi, indicating that cell viability was not affected (data not shown).

Silver induces trafficking of MNK: no effect with cadmium or zinc

We investigated whether other heavy metals with similar chemical properties to copper could induce the trafficking

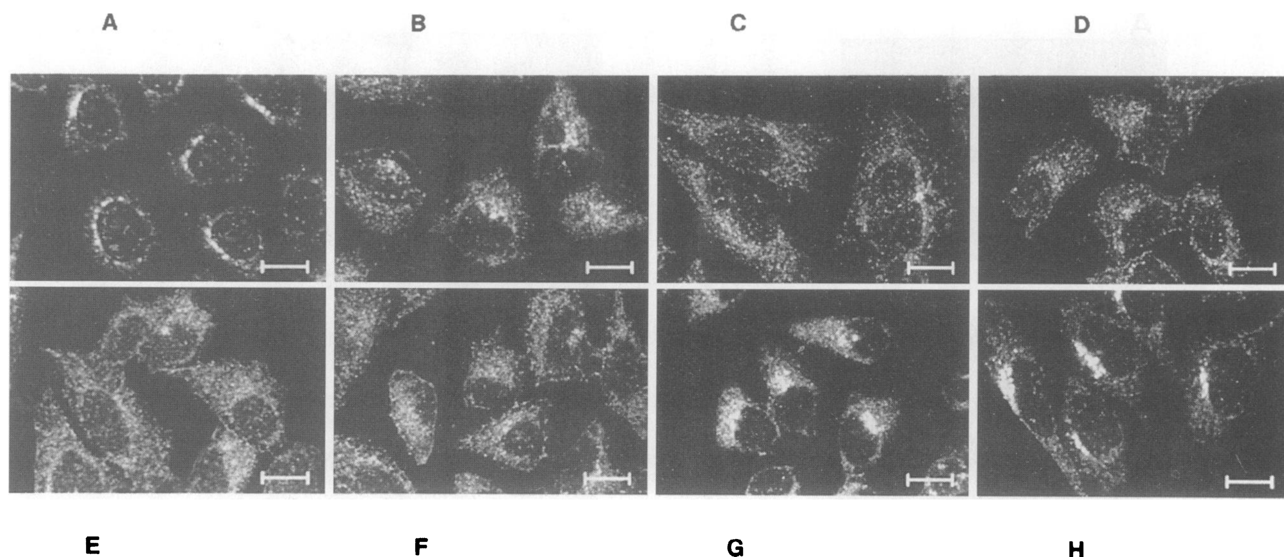

Fig. 7. Time-course analysis of MNK localization in CUR2 cells following the addition and subsequent removal of copper. CUR2 cells grown for 24 h in basal medium (**A**) were incubated in medium supplemented with 189 μM copper for 10 min (**B**), 15 min (**C**), 1 h (**D**) and 24 h (**E**). Copper was withdrawn from CUR2 cells grown for 24 h in 189 μM copper by transferring cells to basal medium for 15 min (**F**), 30 min (**G**) and 1 h (**H**). (A–H) Cells were acetone-fixed and labelled with the MNK antibodies (1:50) followed by FITC-conjugated sheep antibodies to rabbit IgG (1:200). Bars, 10 μm.

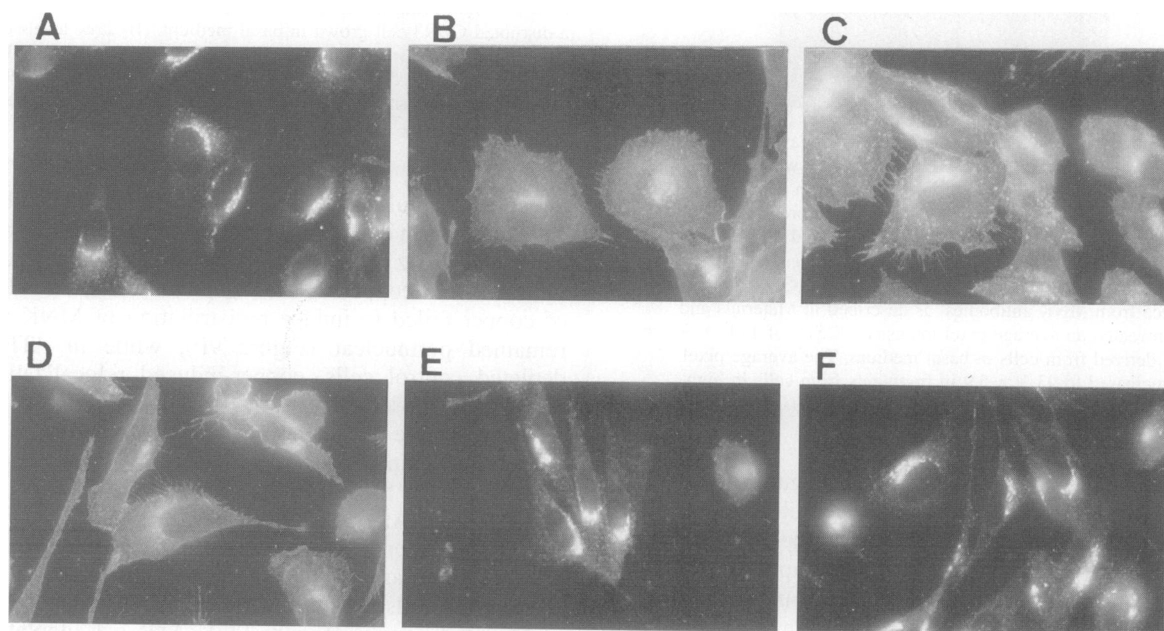

Fig. 8. Analysis of the effect of cycloheximide on MNK trafficking. To test for effects on copper-induced exocytic trafficking of MNK, CUR3 cells which were cultured for 48 h in basal medium (**A**) were pre-incubated for 15 min in basal medium containing cycloheximide (15 μg/ml) and then transferred to medium containing both 189 μM Cu and cycloheximide for 2 h (**B**) or to medium containing 189 μM copper without cycloheximide for 2 h (**C**). To test whether endocytic trafficking of MNK was affected by cycloheximide, CUR3 cells that were grown for 48 h in 189 μM copper (**D**), were firstly pre-incubated for 15 min in medium containing both 189 μM copper and cycloheximide followed by a 2 h incubation in basal medium containing cycloheximide (**E**), or transferred to basal medium without cycloheximide for 2 h (**F**). Cells were then fixed and processed for immunofluorescence using affinity-purified MNK antibodies. Cycloheximide caused 93% inhibition of [^{35}S]methionine incorporation into total cell protein under the conditions described above (data not shown).

of MNK from the Golgi. The metal concentrations used were those at which there is a 50% colony survival of CUR3 cells over a 7-day exposure (methods for colony survival assays as per Camakaris *et al.*, 1995). CUR3 cells were incubated in the presence of silver, zinc or cadmium for 1 h at 37°C (Figure 9). Neither Zn^{2+} (Figure 9B) nor Cd^{2+} (Figure 9C) significantly altered the Golgi localization of MNK from that seen in control cells grown in basal medium (Figure 9A). However, in the presence of Ag^{+}, labelling in the perinuclear region decreased and a punctate cytoplasmic staining was observed (Figure 9D), although the overall effect was less marked than with copper addition (Figure 9E). For each metal tested, increasing the incubation times up to 24 h did not alter the MNK staining patterns from those shown in Figure 9 (data not shown).

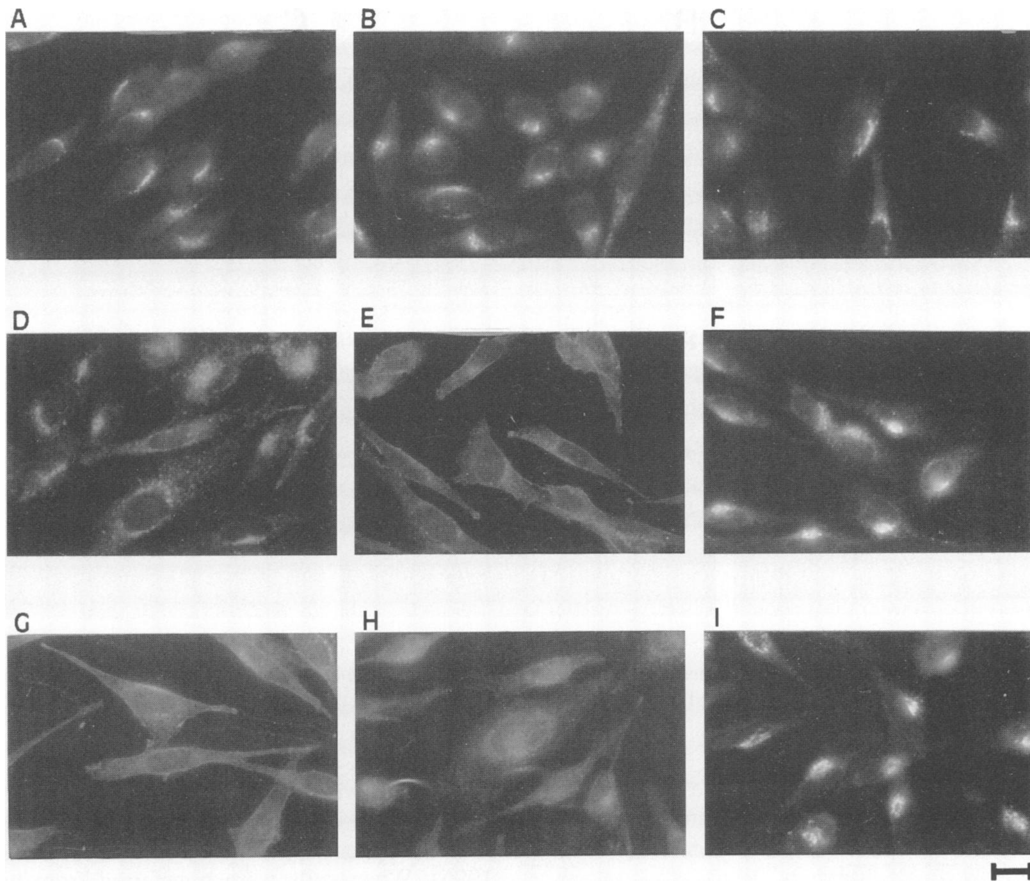

Fig. 9. Analysis of the effect of zinc, cadmium, silver and the effect of ATP-depletion on MNK trafficking. Cells were fixed and MNK detected using MNK antibodies (1:50) after growth in basal medium (**A**), or medium supplemented with 150 μM zinc ($ZnCl_2$) (**B**), 0.2 μM cadmium ($CdCl_2$) (**C**), 4 μM silver ($AgNO_3$) (**D**) or 189 μM copper ($CuCl_2$) (**E**) for 1 h at 37°C. Concurrent experiments were performed testing whether MNK trafficking from the TGN was ATP-dependent. CUR3 cells grown in basal medium were stained with the MNK antibodies (1:50) after a 15 min pre-incubation with DOG/Az followed by 1 h at 37°C in medium with both 189 μM Cu and DOG/Az (**F**). Experiments were performed testing ATP-dependent recycling of MNK from the plasma membrane to the TGN. Immunofluorescence staining was carried out on CUR3 cells grown for 24 h in medium supplemented with 189 μM Cu (**G**), CUR3 grown for 24 h in 189 μM Cu, treated for 15 min with DOG/Az before a 2 h incubation in basal medium containing DOG/Az (**H**). As a control, CUR3 cells were grown for 24 h in medium supplemented with 189 μM Cu followed by a 2 h incubation in basal medium containing no DOG/Az (**I**). Bar, 10 μm for each image.

Evidence for constitutive recycling of MNK

The above results clearly demonstrate an alteration in the localization of MNK in the presence of elevated copper levels and the ability of MNK to recycle from the plasma membrane to the TGN. The possibility arises as to whether there is constitutive recycling between the TGN and the cell surface under basal copper conditions. The endopeptidase, furin, and TGN38 constitutively recycle between the TGN and the plasma membrane and are localized to the TGN by a process of continuous retrieval from the cell surface (Bos *et al.*, 1993; Molloy *et al.*, 1994). The use of drugs which inhibit endosomal recycling results in the accumulation of both these proteins in the endosomal pathway (Chapman and Munro, 1994; Reaves and Banting, 1994). Bafilomycin A_1 is a macrolide antibiotic which blocks endosomal retrieval by specifically inhibiting the activity of the endosomal H^+-ATPase which is necessary for transport from early to late endosomes (Gruenberg and Maxfield, 1995). To test whether MNK continuously recycles via endosomes between the TGN and the plasma membrane even under basal conditions, we investigated whether bafilomycin A_1 could induce a redistribution of MNK. The effects of both chloroquine and NH_4Cl were also tested, as they too inhibit endosomal acidification and arrest the recycling of TGN38 and furin proteins (Chapman and Munro, 1994; Reaves and Banting, 1994). However, unlike bafilomycin A_1, both chloroquine and NH_4Cl are non-specific in their inhibition of endosomal recycling. Chloroquine is a lysosomotrophic amine which has been shown to increase the luminal pH of acidic organelles (De Duve *et al.*, 1974) and NH_4Cl dissipates pH gradients across membranes. As seen in Figure 10B, incubation of CUR3 cells for 3 h in 200 nM bafilomycin A_1 resulted in the redistribution of MNK to vesicular-like structures extending from the Golgi into the cytoplasm. These were clearly distinct from the tight perinuclear location of MNK in untreated cells (Figure 10A). Similar effects on MNK localization were observed when cells were treated with chloroquine (Figure 10C) and NH_4Cl (Figure 10D). The relocalization of MNK by each of these drugs was indistinguishable from the redistribution of TGN38 observed in cells treated with bafilomycin A_1 (Figure 10F compared with 10E). Dispersion of TGN38 was also observed in cells treated with chloroquine and NH_4Cl (data not shown). Hence, the ability of bafilomycin A_1, chloroquine and NH_4Cl to induce an accumulation of MNK in cytoplasmic vesicular-like structures, suggests that MNK is continuously recycling between the cell

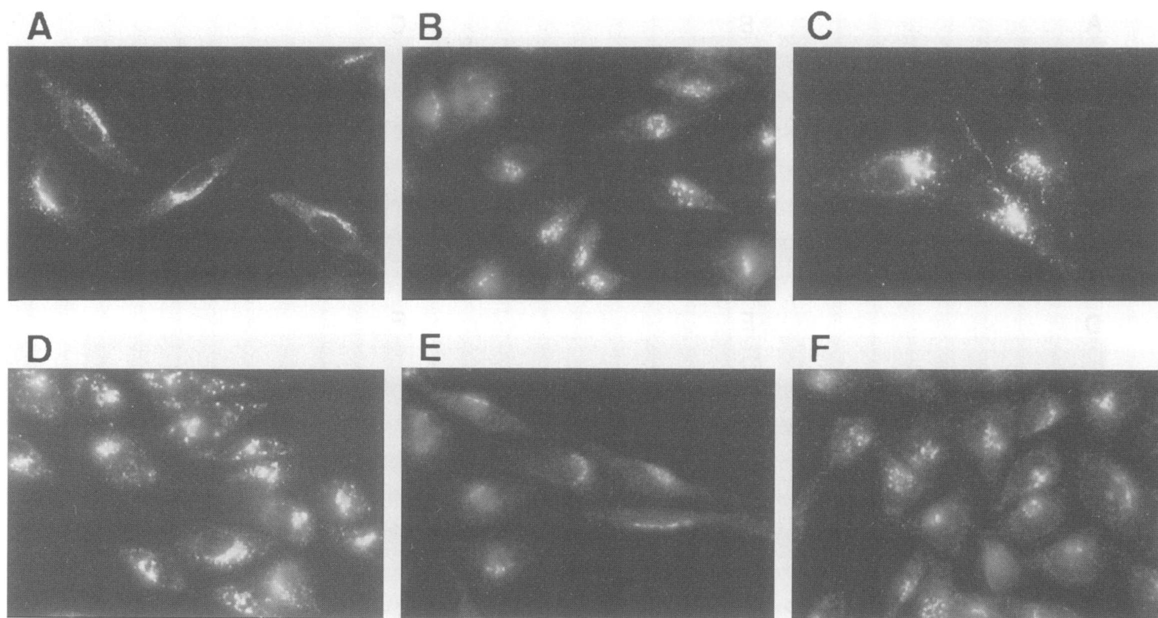

Fig. 10. The effects of bafilomycin A_1, chloroquine and NH_4Cl on the subcellular localization of MNK. CUR3 cells were grown for 3 h in basal medium containing either 200 nM bafilomycin A_1 (**B** and **F**), 200 μM chloroquine (**C**), 50 mM NH_4Cl (**D**) or no added drugs (**A** and **E**). Cells were then fixed and stained using either affinity-purified MNK antibodies (A–D), or TGN38-antiserum (E and F). Primary antibodies were detected using FITC-conjugated sheep antibodies to rabbit IgG.

surface and the Golgi apparatus via endosomes. Therefore, like TGN38 and furin, MNK may be maintained in the TGN by a process of continuous retrieval from the plasma membrane.

Discussion

The results in this paper provide the first indication of how copper homeostasis, involving the MNK P-type ATPase, may be achieved. The intracellular levels of copper must be carefully regulated since an excess of this essential trace element is toxic. MNK has been shown to be crucial for copper homeostasis through its copper efflux activity (Camakaris *et al.*, 1995). A deficiency of MNK, as occurs in cells cultured from patients with Menkes disease, results in the accumulation of excess copper even in basal medium (Goka *et al.*, 1976; Horn, 1976; Camakaris *et al.*, 1980). Furthermore, copper-resistant CHO variants have the capacity for increased efflux of copper by overexpression of MNK (Camakaris *et al.*, 1995).

In the copper-resistant variants cultured in basal medium, the effect of BFA on MNK localization and immunofluorescence studies showing co-localization with the TGN protein, p230, suggested that MNK is localized to the TGN. Although formal confirmation will require immunoelectron microscopy studies, for the purposes of the rest of this discussion we will be referring to a TGN-localization for MNK. In elevated copper, immunofluorescence studies suggested that MNK was trafficked to the plasma membrane. This was demonstrated by the observation of a 5.5-fold increase in MNK in PM lawns derived from CUR3 cells cultured in high-copper media. Immunoelectron microscopic analysis also revealed MNK localization at the plasma membrane in cells cultured in high copper levels. The copper-induced relocation of MNK was not dependent on *de novo* protein synthesis, and occurred within 15 min of copper addition. This is compatible with our observations of rapid increases in copper efflux in the copper-resistant cells following transfer to copper-supplemented media (Camakaris *et al.*, 1995). When cells were transferred from high- to low-copper media, MNK returned to the Golgi, indicating that MNK recycles. Further experiments with inhibitors of endosomal recycling suggested that MNK constitutively recycles via endosomes under basal conditions. Both the endocytic and exocytic trafficking of MNK were found to be dependent on ATP.

In the parental CHO cells, CHO-K1, the immunofluorescence signal was weak and the distribution did not differ significantly between cells cultured in basal or high-copper media, with weak cytoplasmic staining in both conditions. This distribution of MNK in parental CHO cells may reflect the relatively low level of MNK together with the requirement for copper homeostasis under basal conditions. This suggestion is consistent with the proposed recycling model. In the copper-resistant variants which have much higher MNK levels, copper homeostasis in basal medium may only require a small fraction of the available protein to be at the plasma membrane. The CUR cell lines with *MNK* gene amplification were selected for function (copper resistance) as spontaneous variants following stepwise increments in copper concentration of the medium over several months (Camakaris *et al.*, 1995). MNK levels correlate with level of copper resistance and rate of copper efflux across the CHO cell lines (Camakaris *et al.*, 1995). This is compatible with the manner of MNK trafficking being similar at various levels of *MNK* gene expression.

A plausible basis for the copper-induced redistribution of MNK is binding of copper ions to the six putative copper-binding sites in the amino-terminal region of MNK. The closely related copper-transporting ATPase of *Enterococcus hirae* (CopA) (Odermatt *et al.*, 1993) has only one of these amino-terminal copper-binding motifs, suggesting

that the copper-pumping function does not require the six motifs found in MNK. Unlike *MNK*, *copA* is transcriptionally regulated in response to copper (Odermatt and Solioz, 1995). The additional copper-binding motifs in MNK may constitute a copper-sensing domain. As copper concentrations rise in the cytoplasm, these sites may become progressively occupied with copper, thus triggering the exocytic movement of MNK. Retention of MNK in the TGN would need to be disrupted in this process. This may occur if structural alterations in MNK induced by the binding of copper to the copper-binding motifs, disrupt interactions of targeting signals on MNK with adaptor proteins in the TGN and lead to its exocytic movement. Alternatively, given the evidence for constitutive recycling of MNK, copper ions may bind to MNK at the cell surface and prevent recycling, thus shifting the steady-state distribution towards the cell surface. It is possible that both endocytic and exocytic rate constants are altered in the presence of copper, such that there is a net increase in MNK at the cell surface.

Membrane proteins which are endocytosed have targeting signals usually located in the cytoplasmic tails. These are short amino acid sequences which function in internalization, sorting and intracellular retention (Bos *et al.*, 1993; Trowbridge *et al.*, 1993; Schäfer *et al.*, 1995). The two types of sorting signal which have been identified in the peripheral secretory pathway are the tyrosine- and dileucine-based signals, which have roles in various sorting processes involving internalization from the plasma membrane. The tyrosine-based signal has a consensus Y-X-X-hydrophobic amino acid (Collawn *et al.*, 1990), and participates in both the sorting of proteins to endosomes via clathrin-coated pits and protein retention in the TGN (Wilcox *et al.*, 1992; Bos *et al.*, 1993; Humphrey *et al.*, 1993). Dileucine-motifs are involved in the sorting of proteins to lysosomal compartments (Trowbridge *et al.*, 1993) and in the rapid endocytosis and retention of the GLUT4 glucose transporter (Haney *et al.*, 1995). More recently, an autonomous sequence of four acidic amino acids in furin has been shown to be essential in conferring TGN-localization and internalization from the plasma membrane to the TGN (Schäfer *et al.*, 1995; Voorhees *et al.*, 1995). The cytoplasmic domain of the carboxy-terminus of MNK, which is 95 amino acids in length, contains four candidate tyrosine-based signals, three dileucine motifs and a stretch of four acidic amino acids. *In vitro* mutagenesis of these sites is required to ascertain their possible roles in endocytosis and intracellular retention of MNK.

The transition metal ions, Cd^{2+} and Zn^{2+}, did not induce movement of MNK. Silver ions, however, did induce trafficking of MNK from the TGN, although to a less marked extent than copper. The ability of Ag^+ to induce such trafficking is significant as it suggests that Cu^+, rather than Cu^{2+}, induces the trafficking of MNK. Ag^+ can replace Cu^+, but not Cu^{2+}, in proteins with cysteine-based metal-binding sites (Nielson *et al.*, 1985; Casas-Finet *et al.*, 1992). We have found that the copper-resistant CHO cells are also resistant to silver (P.Shen and J.Camakaris, unpublished data), suggesting that Ag^+ can both activate trafficking of MNK, and be effluxed by the protein. The closely related copper-uptake P-type ATPase in *E.hirae*, CopA, may also transport silver (Odermatt *et al.*, 1993).

The copper-regulated trafficking of MNK resembles in some aspects the vesicular trafficking of the glucose uptake transporter, GLUT4. The trafficking of GLUT4 from the TGN and associated tubulovesicular structures to the plasma membrane of fat and muscle cells is induced by increases in the extracellular levels of insulin (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; James *et al.*, 1993). The MNK system is unique, however, in that it mediates the efflux of a potentially toxic ligand, and the ligand itself appears directly to stimulate the trafficking of its own transporter.

What is the biological relevance of MNK recycling between the TGN and the plasma membrane? These two alternative locations of MNK are likely to reflect two important roles of the enzyme. In cultured fibroblasts from Menkes disease patients (MNK loss-of-function) there is a reduced activity of the secreted copper-dependent enzyme, lysyl oxidase (Royce *et al.*, 1980). Hence, in the TGN, MNK may deliver copper to newly synthesized membrane-bound or secreted copper-dependent apoenzymes as they move through the exocytic pathway. Similarly, the closely related copper transporting P-type ATPase, WND, may also have a function in copper transport to enzymes in the Golgi. Copper incorporation into ceruloplasmin during its synthesis in the Golgi is defective in hepatocytes of the Long Evans Cinnamon rat, the bona fide animal model of Wilson disease (Murata *et al.*, 1995; Terada *et al.*, 1995). Furthermore, the copper transporting P-type ATPase in *Saccharomyces cerevisiae*, CCC2, which shows similarity to MNK, is required for the delivery of copper into an extracytosolic compartment to a ceruloplasmin-like oxidase required for iron uptake (Yuan *et al.*, 1995). The additional function of MNK as a copper-efflux pump, following copper-regulated trafficking to the plasma membrane, is consistent with the defect in copper efflux in cultured cells from Menkes patients (Camakaris *et al.*, 1982), and the enhanced efflux observed with copper-resistant CHO variants which overexpress MNK (Camakaris *et al.*, 1995).

In summary, this paper provides the first demonstration of the intracellular localization of MNK and, significantly, demonstrates that the location is altered by changes in copper concentration in the cell environment. The copper-regulated trafficking of MNK is the first known mechanism to explain cellular copper homeostasis and, in addition, is a novel form of regulated protein trafficking. Future work will address whether this copper-regulated trafficking of MNK is a general process found in a variety of cell types, and whether a similar system is utilized for the transport of other heavy metals. It will be of particular interest to identify the structural features of the MNK protein that specify endocytosis, localization, and the copper-regulated trafficking to the plasma membrane.

Materials and methods

Cells and copper concentrations

Cell culturing conditions were as previously described (Camakaris *et al.*, 1995). The copper concentrations chosen for each experiment were those at which there is a measurable increase in copper efflux, while maintaining a high cell viability (Camakaris *et al.*, 1995).

Northern and Western analyses

Extractions and blotting procedures for RNA and protein were performed as described previously (Camakaris *et al.*, 1995).

Drug treatments

Nocodazole and brefeldin A (Sigma, USA) were added to growth medium at final concentrations of 10 μM and 5 μg/ml, respectively. Cells were incubated for indicated times in media containing 50 mM 2-deoxy-D-glucose (Sigma, USA) and 0.02% sodium azide (DOG/Az), based on the procedures used by Lippincott-Schwartz *et al.* (1991) and Reaves and Banting (1992). Cycloheximide was added to growth medium at a concentration of 15 μg/ml. Bafilomycin A_1 (Sigma, USA), chloroquine (Sigma, USA) and NH_4Cl were added to medium to give final concentrations of 200 nM, 200 μM and 50 mM, respectively.

Antibodies

Rabbits were immunized with the amino-terminal 590 amino acids of MNK fused to GST using the pGEX-2T plasmid (Pharmacia, Sweden) as described previously (Camakaris *et al.*, 1995). The pGEX-2T vector, containing an unrelated cDNA (phenylalanine hydroxylase) fused to GST, was expressed in *Escherichia coli*, as previously described (Camakaris *et al.*, 1995), and the soluble proteins were bound to a CNBr-activated Sepharose 4B column (Pharmacia, Sweden). The MNK IgG fraction was prepared by sodium sulphate precipitation (Johnstone and Thorpe, 1987) and passed through the column to remove antibodies against GST and bacterial proteins. Both the MNK antibodies contained in the non-bound fraction and MNK antiserum were used for immunofluorescence studies (as stated in figure legends) and showed no difference in signal specificity. Antibodies against Na^+/K^+ ATPase were raised in rabbits (Callaghan, 1995). Anti-mannosidase-II antibodies were raised in rabbits against the α-subunit (Moremen *et al.*, 1991). TGN38 antibodies were raised in rabbits against the last 22 amino acids of the cytoplasmic C-terminal tail (K.Stanley, unpublished data).

Immunofluorescence microscopy

CHO cells were grown to 70% confluency, as described previously (Camakaris *et al.*, 1995), on glass coverslips and fixed by immersion in acetone at 4°C for 30 s. Cells used in co-localization experiments (Figure 3) were prefixed with 4% formaldehyde in phosphate buffered saline (PBS), pH 7.4, for 15 min and then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Fixed cells were washed in PBS and blocked for 30 min using 1% bovine serum albumin (BSA) in PBS. Cells were incubated for 1 h at room temperature with the primary antibodies diluted in 1% BSA in PBS. Unbound antibodies were removed by washing in PBS, after which cells were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated sheep antibodies to rabbit- or human IgG (Silenus, Australia), or Texas red-conjugated donkey anti-rabbit IgG (Jackson Labs, USA). After a further wash in PBS, the coverslips were mounted onto glass slides using 2.6% DABCO (1,4-diazabicyclo-(2,2,2) octane, Sigma, USA) in 90% glycerol, 10% PBS, pH 8.6. Slides were analysed using a 100× oil objective and confocal laser scanning microscopy (Bio-Rad MRC 1000) (Figures 1, 3 and 7) or using a Reichert–Jung Polyvar microscope (Figures 4, 5, 8, 9 and 10). The addition of 10 μM copper to the antibody-probing buffer did not alter the MNK fluorescence pattern from that observed in the absence of copper supplementation.

Quantitation by immunofluorescence microscopy

Fluorescence intensity was estimated as average pixel intensity of digitized images using laser scanning confocal microscope analysis software (Bio-Rad Laboratories, CA). Fluorescence intensity was measured from equivalent areas containing the perinuclear regions of 12 CUR2 cells from three different fields cultured in both basal- and copper-supplemented medium. Fluorescence intensity in isolated CUR3 plasma membrane lawns was calculated from 12 equivalently sized membrane fragments from three different fields. All images were scaled identically.

Isolation of plasma membrane lawns

This method is based on a one-step procedure previously described (Moore *et al.*, 1987; Heuser, 1989; Lin *et al.*, 1991). Coverslips were coated in poly-L-lysine (0.01% w/v) by immersion and dried before being seeded with cells. Cultured cells on coverslips were washed in PBS and then disrupted by placement under an ultrasonic probe for 1 s (Mk2, MSE Scientific Instruments). Sonicated cells were immediately transferred to 4% paraformaldehyde in PBS and fixed for 15 min before processing for immunofluorescence.

Electron microscopy

CUR3 cells were fixed under lytic conditions (3% formaldehyde, 0.05% glutaraldehyde in 5 mM phosphate buffer, pH 7.4, 10 min, 25°C), washed in 0.1% BSA in 0.1 M phosphate buffer, then reacted with MNK antiserum 1:100) for 1 h at room temperature. After one wash with 0.1% BSA in 0.1 M phosphate buffer, cells were reacted with protein A–5 nm gold (Amersham, UK) and further fixed and processed for electron microscopy as described by Navone *et al.* (1986). Lytic fixation permitted access of reagents to the cytoplasm but did not preserve Golgi structures.

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