Expression of Menkes disease gene in mammary carcinoma cells

M. Leigh ACKLAND *[1], E. Jean CORNISH*, Jenny A. PAYNTER†, Andrew GRIMES†, Agnes MICHALCZYK*
and Julian F. B. MERCER†

School of Biological and Chemical Sciences, Deakin University, Rusden Campus, 662 Blackburn Road, Clayton, Victoria 3168, Australia, and †Murdoch Institute, Royal Children's Hospital, Flemington Road, Parkville, Victoria, 3052 Australia

Two P-type ATPases, MNK and WND were recently shown to be defective in the human disorders of copper transport, Menkes disease and Wilson disease respectively. These proteins are important in copper homeostasis but their full physiological function has not been established. This study uses the human breast carcinoma line, PMC42, to investigate copper transport in the mammary gland. Northern blot analysis indicated that both MNK and WND mRNA are expressed in these cells. Western blot analysis with an MNK-specific antibody demonstrated a band of approx. 178 kDa, close to the expected size of 163 kDa. Treatment of PMC42 cells with lactational hormones (oestrogen and progesterone for 3 days followed by dexamethasone, insulin and prolactin for a further 3 days) did not produce an obvious increase in MNK expression as measured by Northern and Western blots. By using indirect immunofluorescence with the MNK antibody, the intracellular distribution of MNK was found to be predominantly perinuclear, consistent with Golgi localization. Punctate staining was also seen in a smaller proportion of cells, suggesting that some MNK is associated with endosomes. Treatment of PMC42 cells with lactational hormones increased the intensity of the perinuclear and punctate fluorescence. Exposure of cells to 100 mM copper resulted in the dispersion of the fluorescence towards the periphery of the cell. The results suggest a role for MNK in the secretion of copper into milk and that PMC42 cells are a valuable model for investigating the detailed cellular function of MNK and WND.

INTRODUCTION

Copper is an essential element that functions as a cofactor of a number of important oxidative enzymes such as cytochrome *c* oxidase, dopamine β-hydroxylase and lysyl oxidase. Deficiency in copper is particularly devastating to the young mammal and is graphically demonstrated in the human genetic copper-deficiency disorder, Menkes disease. Affected boys rarely survive beyond 3 years of age and suffer from symptoms that include severe neuronal degeneration and arterial and bone defects [1]. During the neonatal period, copper must be supplied to the growing mammal in milk. Little is known, however, of the cellular processes by which copper is secreted into milk [2].

Two novel human genes involved in copper transport have been isolated. The first is affected in the human genetic disorder of copper transport, Menkes disease [3–5]; the second is affected in another disorder of copper transport, Wilson disease [6–8]. Both the Menkes and Wilson disease genes encode similar proteins, MNK for the predicted product of *MNK*, the Menkes gene, and WND for the predicted product of the Wilson gene, *WND*. These proteins are members of the family of P-type ATPases (MNK and WND are also termed ATP7A and ATP7B respectively), transmembrane proteins that link the hydrolysis of ATP to cation transport [9]. The MNK and WND proteins have been detected in a variety of human tissues. MNK mRNA seems to be expressed primarily in non-hepatic tissues [10], whereas WND is expressed mainly in the liver, with some expression in kidney, brain and lung [6,7]. Recent immunofluorescent work with a polyclonal antibody to WND has shown both a canalicular expression of WND in human liver tissue and also a more uniform cytoplasmic distribution of this protein in this tissue [11].

The full biological role of these novel molecules has not been clearly established. The features of Menkes disease can be explained if MNK acts as a copper efflux pump and this has been confirmed by copper transport studies in copper-resistant Chinese hamster ovary cells. In these copper-resistant variants, the Menkes gene homologue has been amplified and MNK increased up to 100-fold compared with the copper-sensitive parental line. The increased MNK allowed the resistant cells to transport copper out of the cell more rapidly [12]. Recently, MNK has been localized to the *trans*-Golgi network in these cells and, most significantly, when cells are exposed to high concentrations of copper, MNK distribution changes markedly and is found associated with cytoplasmic vesicles and on the plasma membrane [13]. It was proposed that this relocalization of MNK by copper is important for the maintenance of cellular homeostasis [13], but so far the localization of MNK in other cell types has not been reported and the copper-induced relocalization has been reported only in copper-resistant cells.

No reports have appeared concerning the expression of MNK or WND in mammary tissue. The efflux role of MNK suggest it is likely to play a part in delivering copper to milk. WND might also be involved in this process because a mouse mutant that produces copper-deficient milk, the toxic-milk mouse [14], has been shown to have a mutation in the murine WND homologue [15]. To study the expression of WND and MNK in mammary-derived tissue, a unique human breast carcinoma line, PMC42, was chosen. This line is thought to derive from a stem cell that has retained the ability to differentiate into cell types similar to those found in the mammary gland [16].

Ultrastructural studies have shown PMC42 cells with characteristics of secretory cells, containing lipid granules, swollen endoplasmic reticulum and secretory vesicles, whereas others have characteristics of myoepithelial cells such as contractile fibrils [16]. Growth of PMC42 cells can be stimulated by β-oestradiol, progesterone, insulin and hydrocortisone, and prolactin induces the formation of lipid droplets [17,18]. These cells

Abbreviations used: DTAF, dichlorotriazinyl aminofluorescein; MNK, WND, P-type ATPases defective in Menkes disease and Wilson disease respectively.

[1] To whom correspondence should be addressed.

are thus a valuable model system for investigating the role of MNK and WND in mammary tissue, the intracellular location of these proteins in different cell types and the influence of hormones on the expression of these genes.

In this paper we show that both MNK and WND are expressed in PMC42 cells and that MNK is located in the Golgi and possibly endosomes. The distribution of MNK is altered in the presence of copper, suggesting a mechanism by which this ATPase could deliver copper to milk.

MATERIALS AND METHODS

Cells and culture conditions

PMC42 cells, a gift from Dr. R. Whitehead, were originally derived from a pleural effusion from a woman with metastatic breast cancer [16]. Cells were grown in Nunclon 25 cm^2 or 80 cm^2 culture flasks in RPMI 1640 (Trace Biosciences) supplemented with 10% (v/v) fetal bovine serum (CSL). The cells were split when confluent by using 0.025% trypsin–versene solution (Sigma). Cultures were viewed with an Olympus CK2 inverted phase-contrast microscope.

Hormone treatment

To determine whether expression of MNK and WND might be influenced by lactation, PMC42 cells were treated with a mixture of hormones that mimicked the pattern of hormones experienced by the mammary gland during lactation. Cells were grown to pre-confluency in RPMI 1640 with 10% (v/v) fetal bovine serum. On day 0, β-oestradiol (10 nM; Sigma) and progesterone (500 nM; Sigma), dissolved in ethanol, were added to cultures. The control cultures received an equal volume of ethanol alone. On day 3 the culture medium was replaced with medium containing dexamethasone sodium phosphate (1 μg/ml; MSD), insulin (0.6 μg/ml; Novo Nordisk) and prolactin (luteotropic hormone, 200 ng/ml; Sigma) in aqueous solution. On day 6 the culture medium containing the three hormones given on day 3 was renewed and cells were harvested 2 h later. For copper treatment, cells were exposed to 100 μM copper acetate in RPMI 1640 medium with 10% (v/v) fetal bovine serum for 30 min before harvesting.

MNK antiserum preparation

A 1.8 kb cDNA encoding the six copper-binding domains of MNK, from nt 149 to nt 1917 by the Vulpe sequence numbering [5], was subcloned into pGEX-2T (Pharmacia) and expressed as a 94 kDa glutathione S-transferase fusion protein. *Escherichia coli* pellets were isolated after induction as described by the manufacturers and the fusion protein was extracted by sonication in 50 mM Tris/HCl (pH 7.5)/100 mM NaCl/10 mM dithiothreitol/1 mM PMSF/1% (v/v) sarkosyl. Cell debris was removed by centrifugation and the product was purified by SDS/PAGE [7% (w/v) gel] with a Bio-Rad Prepcell. Antibodies were raised in a rabbit by intramuscular injection of 100 μg of purified protein in 500 μl of 25 mM Tris/192 mM glycine mixed with 500 μl of Freund's complete adjuvant for the first injection and incomplete adjuvant for all subsequent injections. An IgG fraction was obtained from the crude serum by precipitation with sodium sulphate [19]. Affinity purification was performed by passage of the IgG fraction through a Sepharose column that contained the same region of MNK without the glutathione S-transferase. This protein was obtained by using the expression vector pQE30 (six-His tag; Qiagen) and purified with Ni resin; it was coupled to CNBr-Sepharose (Pharmacia) to prepare the affinity absorbant. Bound antibodies were eluted with 0.1 M glycine, pH 2.5. Purified antiserum was stored at -20 °C at a concentration of 60 μg/ml. To determine the degree of cross-reactivity of the MNK antiserum to the metal-binding domains of the closely related Wilson disease gene product, WND [6], the WND metal-binding regions of mouse WND were expressed from a mouse cDNA [15] with the pQE30 system and purified by Ni-affinity chromatography. The reactivity of the MNK antiserum to the WND product was found to be less than 1% of MNK by using both Western blots and an ELISA assay (A. Grimes, unpublished work).

RNA isolation and Northern blots

RNA, isolated from PMC42 cells with an RNeasy kit from Qiagen, was fractionated on 1% (w/v) agarose gels with 0.66 M formaldehyde and transferred to Hybond N^+ membranes. Blots were probed with a combination of cDNA clones c3.13 and c3E for MNK, which encode the N-terminal region of human MNK [4]; WND cDNA clones Wc1.c8 and c1.gb10 for WND mRNA [6] (kindly provided by Diane Cox); and glyceraldehyde-3-phosphate dehydrogenase cDNA [20]. Inserts were labelled with [^{32}P]dCTP by the random primer extension method with a kit supplied by Boehringer Mannheim. Filters were washed in 0.2 × SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS at 65 °C. The filters were exposed to Biomax MS film (Kodak) at -70 °C.

Western Blot analysis

PMC42 cells grown in 80 cm^2 plastic flasks were washed twice with PBS (15 ml at room temperature); cells were scraped off in 6 ml of PBS and two 3 ml aliquots were centrifuged at 3000 ***g*** for 5 min at room temperature. The cell pellet was resuspended in PBS or Tris buffer, pH 6.8 (300–500 μl), containing 20 mg/ml SDS, 1 mg/ml Mega 10, 2 μg/ml DNase, 5 mM 2-mercaptoethanol and the following protease inhibitors: 2 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride, 25 μg/ml aprotonin, 5 μg/ml pepstatin A and 5 μg/ml leupeptin (Sigma). Cells were lysed by repeated passages through a 21-gauge needle followed by 40 strokes of a Dounce homogenizer. The extract (20–60 μg of protein) was fractionated by SDS/PAGE [7.5% (w/v) gel] with a Bio-Rad Mini Protean Gel system in accordance with the manufacturer's instructions. Proteins were transferred to nitrocellulose membranes at 4 mA/cm^2 for 120 min with a Bio-Rad Mini Trans-Blot in 25 mM Tris/192 mM glycine. After blocking with 1% (w/v) casein in TBST (0.05 M Tris/0.15 M NaCl/0.1% Tween-20), the membrane was exposed to the primary antibody at 1/50 dilution in blocking buffer for 2 h, then washed in blocking buffer (four washes of 15 min each). MNK was detected by using a 1:1000 dilution of a fluorescein-conjugated goat anti-rabbit antibody and a Boehringer chemiluminescent detection kit (Catalogue no. 1500798) in accordance with the manufacturer's instructions. The membranes were placed in contact with Kodak XAR X-ray film for 30–60 s.

Indirect immunofluorescence

PMC42 cells were seeded on 10 mm diameter coverslips (Mediglass) in 25 mm Petri dishes (Greiner) at a density of 10^5 cells per dish and grown to pre-confluency before being washed in PBS and fixed for 15 min in 4% (w/v) paraformaldehyde. The cells were blocked with 1% (w/v) BSA in PBS and permeabilized in 0.1% (w/v) Triton X-100 in PBS. First-antibody dilutions (1:20 for MNK and 1:1000 for control rabbit antiserum and pre-immune serum) were applied to cells for 2 h at room

temperature. After three washes with PBS, the second antibody, dichlorotriazinyl aminofluorescein (DTAF)-conjugated Affini-Pure donkey anti-(rabbit IgG) (Jackson Immunoresearch Laboratories), at 1:200 dilution in 1 % (w/v) BSA in PBS, was applied for 2 h. The coverslips were inverted on a glass slide with a drop of 1,4-diazobicyclo[2,2,2]octane (DABCO) (Sigma) and epifluorescence was viewed with an Olympus AX70 Provis microscope with a U-MWB filter cube and a PlanApo 60X 1.4 oil objective.

RESULTS

PMC42 cells express both MNK and WND mRNA

Northern blots of RNA isolated from PMC42 cells were probed with an MNK cDNA, which detected a band at 8.5 kb, the same size as the MNK mRNA previously reported (Figure 1A) [4]. The signal intensity from cells grown in the presence of the lactation hormones (Figure 1A, lanes 4–6) was not significantly higher than two of the control cells samples (Figure 1A, lanes 1–3), so there was no clear effect of hormone treatment on MNK mRNA. After stripping of the MNK signal, the filter was probed with human WND cDNA and a signal of similar strength to the MNK was detected at approx. 7.5 kb (Figure 1C), corresponding to the reported size of WND mRNA [6]. As with MNK, the hormone treatment did not produce a striking change in the WND signal. The relative amount of mRNA loaded on the gel was estimated by probing with a cDNA for the housekeeping gene GAPDH (Figure 1B) and from the ethidium bromide-stained gel (Figure 1D).

Western blot analysis of MNK expression

PMC42 cell extracts were fractionated on 7.5 % polyacrylamide gels and blotted on nitrocellulose as described in the Materials and methods section. The MNK band was identified with an affinity-purified antiserum and a chemiluminescence detection system. A band of approx. 178 kDa was observed (Figure 2A), close to the expected size (163 kDa) on the basis of the predicted coding sequence [5]. No bands were detected when pre-immune serum was used (results not shown). As with the mRNA there was no clear effect of the hormone treatment on the levels of MNK in the extracts (compare lanes 1–3 with lanes 4–6 in Figure 2A). The Coomassie Blue-stained protein bands indicate the evenness of loading on the gel (Figure 2B). Treatment of cells with 100 μM copper for 30 min did not alter the intensity of the 178 kDa band (results not shown).

Intracellular localization of MNK

The intracellular distribution of MNK protein in the cells was assessed by indirect immunofluorescence. Relative to the control

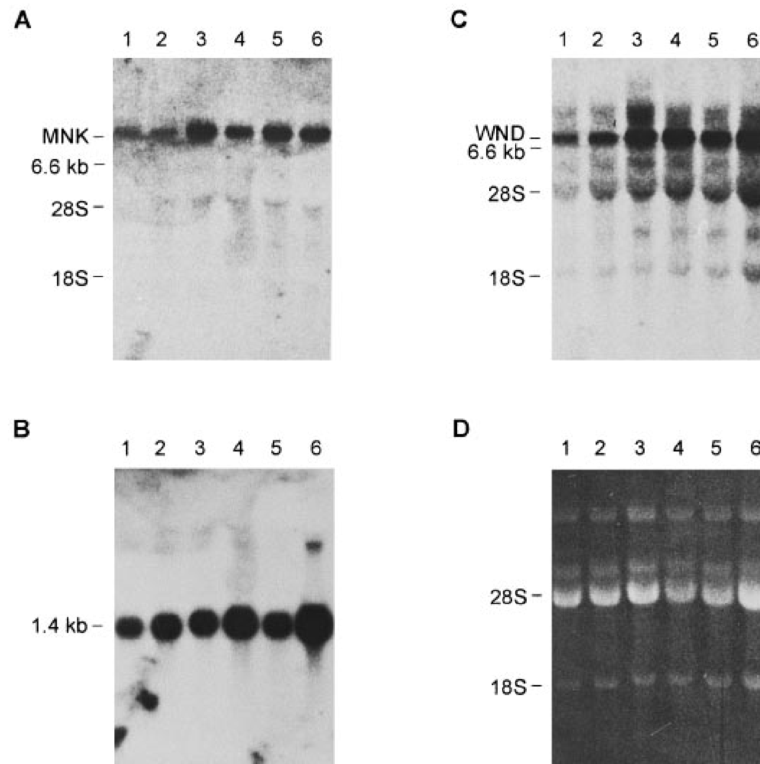

Figure 1 Northern blot analysis of RNA prepared from PMC42 cells

(**A**) Hybridization with human Menkes cDNA clones c3.13 and c3E [4] demonstrates an 8.5 kb band above the 6.6 kb marker. (**C**) Hybridization with Wilson cDNA clones Wc1.c8 and Wc1.gb10 [6] shows a band of approx. 7 kb. The evenness of loading of RNA is demonstrated by hybridization with a glyceraldehyde-3-phosphate dehydrogenase probe (**B**) and by comparison with rRNA stained with ethidium bromide (**D**). Lanes 1–3, RNA from three different batches of control cells; lanes 4–6, RNA from three different batches of hormone-treated cells (β-oestradiol, progesterone, dexamethasone, insulin and prolactin as described in the Materials and methods section). Approx. 10 μg of RNA was applied to each lane. Bands were detected by autoradiography with Kodak Biomax MS film and exposed for 5 days at −70 °C with a Biomax MS screen.

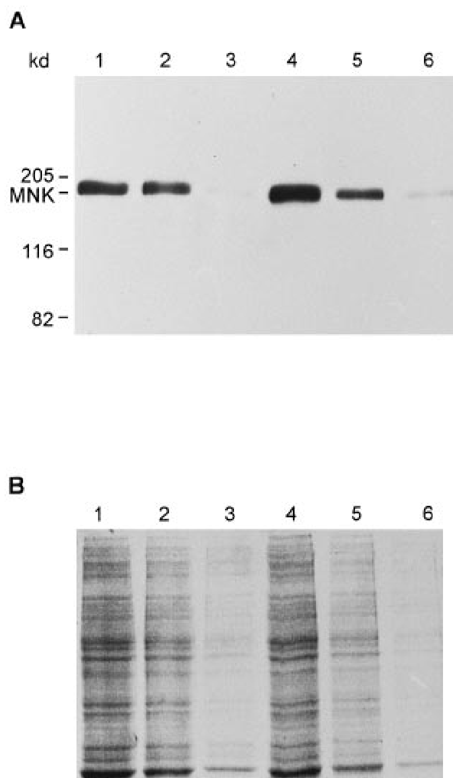

Figure 2 Western blot analysis of PMC42 extracts

Cells were grown in medium with no added hormones or with hormones as described in the legend to Figure 1. Lanes 1–3 were loaded with 60, 40 and 20 μg of protein from control cells respectively. Lanes 4–6 were loaded with 60, 40 and 20 μg of protein from hormone-treated cultures respectively. (**A**) A membrane from one of 45 similar experiments was probed with affinity-purified MNK antibody (1 : 50) and bands were detected with a horseradish peroxidase chemiluminescence system as described in the Materials and methods section. The positions of molecular mass markers (in kDa) are shown at the left. (**B**) Coomassie Blue stain of the same gel as in (**A**).

treated with pre-immune serum (Figure 3A), a perinuclear fluorescent signal was detected in over 90 % of cells grown without added lactational hormones (Figure 3B). There was considerable variation in the signal intensity between cells. In some cells a more dispersed punctate pattern was evident (Figure 3C). Cultures of PMC42 cells usually contained a small proportion of multinucleated cells (between 2 % and 5 % of the total cell population); the fluorescence in these was similar in intensity to the singly nucleated cells. The perinuclear fluorescence is consistent with *trans*-Golgi localization reported for MNK in Chinese hamster ovary cells [13], and the punctate staining suggests that some MNK might be located on endosomes.

Cells treated with the hormone mixture as described in the Materials and methods section showed a similar distribution of staining to that in the control cells (compare Figure 4A with Figure 3B). Although not marked in Figure 4(A), the intensity of staining seemed greater in the hormone-treated cells, suggesting that more MNK might be produced. The punctate staining extending into the cytoplasm was also evident. The addition of $CuCl_2$ (100 μM Cu^{2+} for 30 min) to control cells resulted in a marked alteration in the staining pattern, with a dispersion of the perinuclear fluorescence towards the periphery of the cells (Figure 4B). This effect was also evident in the hormone-treated cells grown in high copper concentrations (Figure 4C). The dispersion of the perinuclear fluorescence by copper resembles that seen in copper-resistant Chinese hamster ovary cells [13].

We were unable to detect WND protein in PMC42 cells by using an antibody raised to a 266-residue portion of mouse ATP7B. Although we expected the WND antibody to cross-react with the human protein, we did not obtain a positive signal from human liver extracts on a Western blot, in contrast with the positive mouse liver control.

DISCUSSION

In this investigation the expression of the copper transporters MNK and WND in a cultured mammary carcinoma line, PMC42, was analysed. The Northern blots demonstrate that both genes are expressed, and in similar quantities. Treatment of the cells with a mixture of hormones that induce lactation (β-oestradiol, progesterone, dexamethasone, insulin and prolactin) did not cause any major change in the levels of MNK and WND mRNA and this was confirmed by the Western blots with the MNK antiserum and by immunocytochemistry. Although not marked, the immunofluorescence analysis suggested some increase in the signal intensity in the hormone-treated cells, suggesting that the hormone treatment might increase the amount of MNK; however, to establish this would require more quantitative analysis than was undertaken in this investigation.

The MNK-specific antiserum allowed us to investigate the intracellular localization of MNK. Knowledge of the localization of this copper ATPase is vital to an understanding of its role in copper transport. MNK has been localized to the *trans*-Golgi network of copper-resistant CHO cells in which the MNK gene has been amplified [13]. Our results suggest that in PMC42 cells the primary location of MNK is intracellular and the perinuclear position is consistent with localization to the Golgi apparatus; however, further studies are needed to confirm this and to establish whether MNK is in the Golgi stacks or the *trans*-Golgi network. The punctate staining, which was evident in some cells (e.g. in Figure 3C) and was the predominant pattern in a minority of cells, suggests that some MNK is to be found in endosomes. These observations are consistent with the model of Petris et al. [13], in which MNK is recycled from the *trans*-Golgi network to the plasma membrane and returned via endosomes.

When cells were treated with elevated copper concentrations for 30 min, the localization of MNK became more diffuse. A similar result was found by Petris et al. [13] in copper-resistant CHO cells; they proposed that copper affects the normal recycling of MNK between the *trans*-Golgi network and the plasma membrane, such that more MNK is located on the plasma membrane to allow the efflux of excess copper. Our results are consistent with this proposal, suggesting that this copper effect is not simply a phenomenon induced by the selection of cells for resistance to copper, and indeed might be the common mechanism of MNK action in many cell types.

The vesicular localization and the observation that MNK is relocalized by copper in PMC42 cells suggest a possible model for copper delivery to milk. MNK might pump copper either into vesicles at the *trans*-Golgi network, which are subsequently transported to the plasma membrane and release their contents into the milk, or by direct efflux through MNK located at the plasma membrane. We intend to explore the former possibility by co-localization studies of MNK with specific milk proteins such as α-lactalbumin, lactoferrin and β-casein, which have known intracellular pathways from the Golgi via secretory vesicles to the plasma membrane.

Although we were able to detect MNK protein with an antibody raised against a mouse protein, it seemed that the

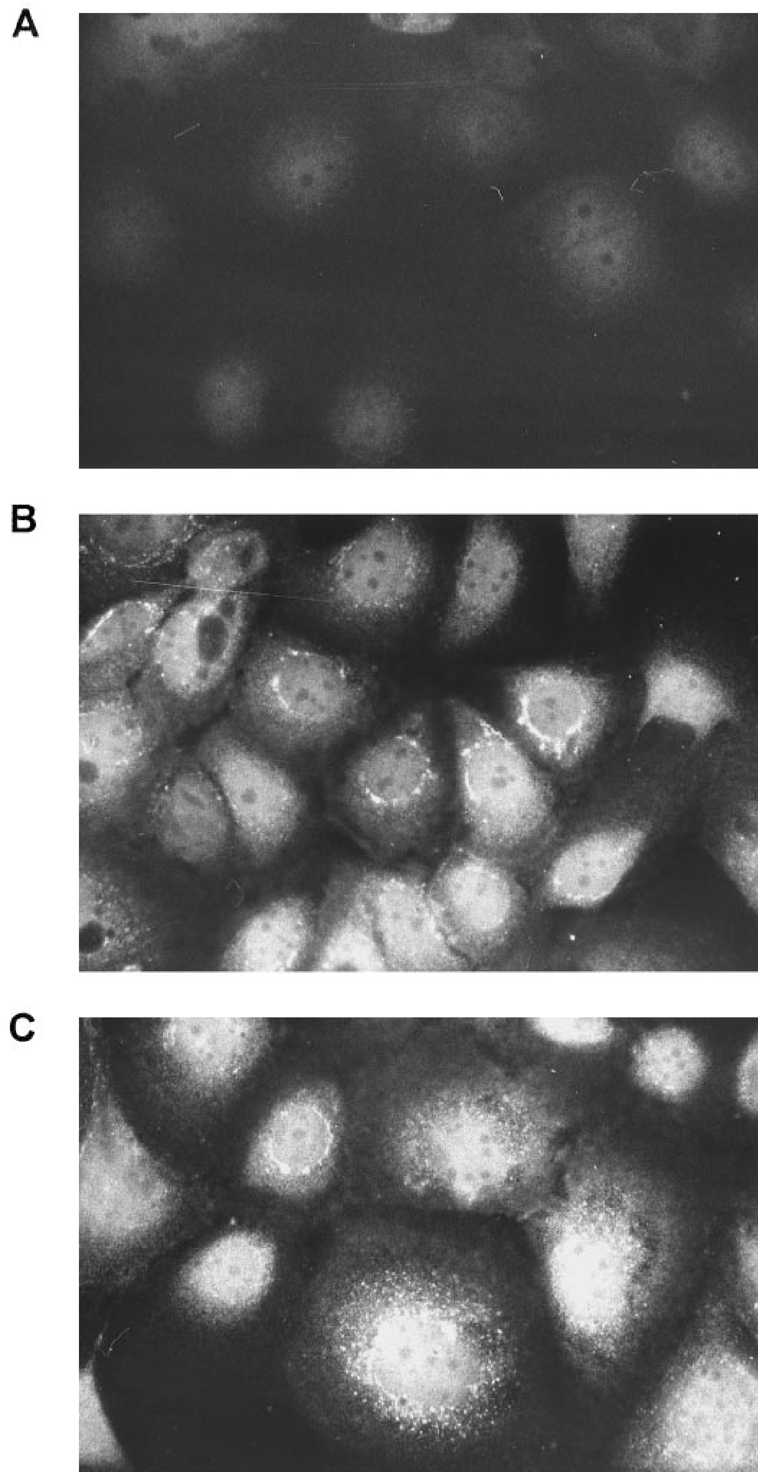

Figure 3 Localization of MNK protein in PMC42 cells

(**A**) Cells were grown in medium with no added hormones, fixed in paraformaldehyde and treated with pre-immune serum (1:1000) followed by DTAF-conjugated anti-(rabbit IgG) (1:200) for 2 h. (**B**,**C**) Cells were grown in medium with no hormones, fixed in paraformaldehyde and treated with MNK affinity-purified (1:20) antiserum overnight followed by DTAF-conjugated anti-(rabbit IgG) (1:200) for 2 h. Epifluorescence was observed with a ×60 PlanApo oil objective. The above is one of 18 similar experiments. Magnification, ×510.

WND antibody raised against the mouse ATP7B did not cross-react with the human protein. We were therefore unable to determine whether WND was present in PMC42 cells and where it was located. When an antibody to human WND becomes available, it will be of interest to determine whether WND is expressed in the same cell types as MNK. Previous reports

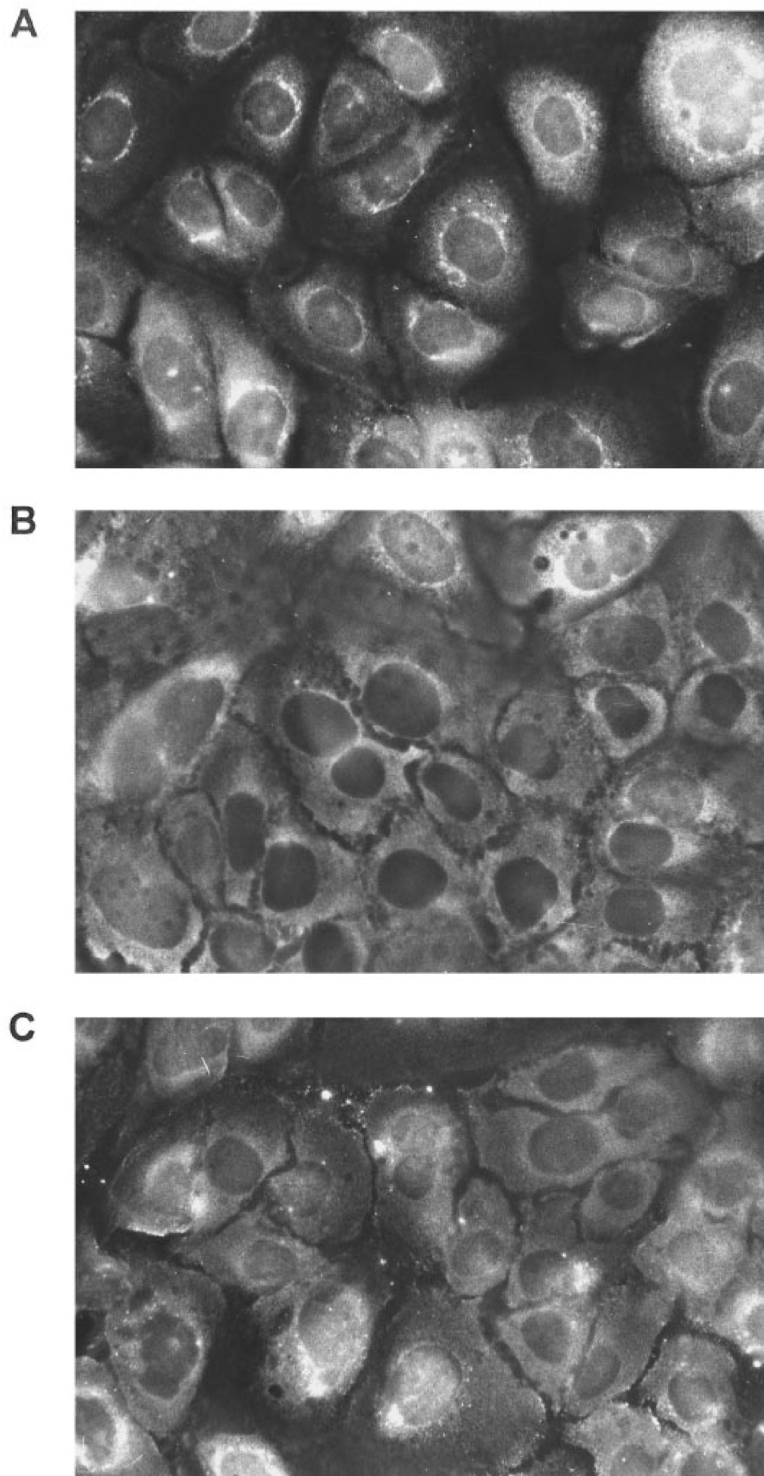

Figure 4 Effect of hormones and copper treatment on MNK localization in PMC42 cells

(**A**) Cells were grown in medium with hormones as described in the Materials and methods section. (**B**) Cells were grown in medium without hormones and then treated with copper (100 μM for 30 min). (**C**) Cells were grown in medium with hormones as described in the Materials and methods section and then treated with copper (100 μM for 30 min). Cells were fixed with paraformaldehyde and incubated overnight with MNK affinity-purified antibody (1:20). Detection was with DTAF-conjugated anti-(rabbit IgG). Epifluorescence was observed with a $\times 60$ PlanApo oil objective. Similar experiments were repeated 12 times. Magnification, $\times 510$.

suggest that some tissues such as kidney and placenta express both MNK and WND [6,7,10,15], but there is no information about whether the same cells express both genes, and if so whether they are both localized to the *trans*-Golgi network.

If these similar copper ATPases prove to be expressed in the same cell, then they might have distinct copper transport

functions within that cell. The major site of WND expression is the liver, where it is likely to have a role in both the biliary excretion of copper and the delivery of copper to ceruloplasmin. Ceruloplasmin expression has been demonstrated in epithelial cells lining the mammary gland alveolar ducts of the rat mammary gland and in human breast cancer tissue [21]. Thus it is conceivable that if WND is expressed in the mammary gland it might be involved in the delivery of copper to apoceruloplasmin; indeed this might be the primary function of this copper transporter in that tissue. We suggest, however, that MNK is likely to be the principal means by which copper is transported to milk.

In conclusion, we have shown that both MNK and WND mRNA are expressed in a mammary carcinoma line and MNK protein was detected in these cells. MNK was found predominantly within the cell in a perinuclear location, most probably in the Golgi apparatus, but also in punctate-staining endosomal-like structures. These results suggest that MNK might be involved in copper secretion into milk.

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