Correction of the Copper Transport Defect of Menkes Patient Fibroblasts by Expression of the Menkes and Wilson ATPases*

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Menkes' disease is a fatal, X-linked, copper deficiency disorder that results from defective copper efflux from intestinal cells and inadequate copper delivery to other tissues, leading to deficiencies of critical copper-dependent enzymes. Wilson's disease is an autosomal inherited, copper toxicosis disorder resulting from defective biliary excretion of copper, which leads to copper accumulation in the liver. The ATP7A and ATP7B genes that are defective in patients with Menkes' and Wilson's diseases, respectively, encode transmembrane, P-type ATPase proteins (ATP7A or MNK and ATP7B or WND, respectively) that function to translocate copper across cellular membranes. In this study, the cDNAs derived from a normal human ATP7A gene and the murine ATP7B homologue, Atp7b, were separately transfected into an immortalized fibroblast cell line obtained from a Menkes' disease patient. Both MNK and WND expressed from plasmid constructs were able to correct the copper accumulation and copper retention phenotype of these cells. However, the two proteins responded differently to elevated extracellular copper levels. Although MNK showed copper-induced trafficking from the trans-Golgi network to the plasma membrane, in the same cell line the intracellular location of WND did not appear to be affected by elevated copper.

Copper homeostasis is important because copper is required in trace amounts for the normal functioning of many biological processes but is toxic in excess due to its redox properties (1, 2). The importance of copper homeostatic mechanisms is evident in copper transport disorders such as Menkes' and Wilson's diseases (1), where there is a breakdown of these processes. The isolation of the genes that are defective in Menkes' (ATP7A) (3–5) and Wilson's (ATP7B) (6–8) diseases and the identification of the encoded proteins ATP7A (MNK) and ATP7B (WND), respectively, as key components of the copper transport pathway represented a significant advance in our understanding of the molecular basis of two important inherited diseases of humans, as well as the biology of copper transport.

In Menkes' disease, which is a copper deficiency disorder, many of the biochemical and clinical symptoms are attributable to reduced levels of critical copper-requiring enzymes. The clinical features of Wilson's disease stem from an accumulation of hepatic copper to toxic levels (1). Both MNK and WND belong to the family of cation-transporting P-type ATPase proteins and function to transport copper across cellular membranes. MNK is expressed in most tissues except the liver (3, 5), whereas WND is expressed predominantly in the liver and at lower levels in a number of other tissues (6, 8). Therefore, despite their high degree of similarity at the protein level (9), at the level of the whole organism these two proteins appear to have distinct roles in copper transport and homeostasis (10).

A wide range of mutations within the ATP7A and ATP7B genes have been described in patients with Menkes' (11–13) and Wilson's diseases (13, 14), respectively. In addition, patients with these disorders exhibit a diversity of phenotypes with respect to disease severity (13, 15), and there is currently no useful genotype/phenotype correlation. There is also no formal evidence that mutations in these genes are solely responsible for the copper transport defect and the diverse phenotypes of Menkes' and Wilson's disease patients. A diagnostic feature of Menkes' disease is that cultured cells from patients hyperaccumulate copper (16–18) and show increased retention of $^{64}$Cu, which provides evidence for defective copper efflux (13, 19). To determine whether the proteins expressed from the MNK and WND cDNAs could correct the abnormal copper metabolism of such cells, a human skin fibroblast cell strain, Me32a, from a patient with classical Menkes' disease was immortalized. Transfection of the cDNAs encoding the MNK and WND proteins into these cells led to restoration of copper efflux by these cells. However, although both proteins were localized to a perinuclear region that was consistent with the TGN in basal copper levels, their intracellular location differed in the presence of elevated copper levels. This report represents the first demonstration of the correction of the copper accumulation and retention phenotype of cultured fibroblasts from a Menkes patient by the Menkes protein, as well as by the closely related Wilson protein.

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1 The abbreviations used are: TGN, trans-Golgi network; BME, Eagle's basal medium; CHO, Chinese hamster ovary; FCS, fetal calf serum; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; kb, kilobase(s); LEC, Long-Evans Cinnamon.

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**Restoration of Copper Transport by Menkes Fibroblasts**

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Transfection Experiments**—Cells were maintained in basal medium (1.5 μM copper), which consisted of Eagle's basal medium (BME, Trace Biosciences) supplemented with 10% fetal calf serum (FCS, Trace Biosciences), l-proline at a final concentration of 20 μM, and 0.2% (w/v) bicarbonate. The Me32a primary cell strain was derived from a skin biopsy that was taken from a patient with classical Menkes' disease and showed a copper accumulation phenotype typical of this disease. Immortalization of this cell line by SV40 gene transfer was carried out as described previously (20) except that the plasmid pSV-T22 was used, which contains a single nucleotide substitution in codon 203 (His → Gln) that renders the large T antigen replication incompetent. The resulting cell line was designated Me32a-T22/2L, and DNA fingerprinting analysis confirmed that this line was derived from the original parental cell strain. The SV40-immortalized normal human fibroblasts GM639, GM847, and GM2069 SV9 (Coriell Institute, Camden, NJ) were employed as controls.

The immortalized cell line, Me32a-T22/2L, was transfected with either the expression vector (pCMB77) alone or with pCMB117 or pCMB98, both of which carry the human MNK (21) and mouse WND4 (22) cDNAs, respectively. Liposome-mediated transfection was carried out using Superfect reagent (Qiagen) and recommended protocols, followed by selection in G418 (Life Technologies Inc.) for 2 weeks. Screening of G418-resistant transfectants for those that expressed the MNK or WND proteins was carried out by indirect immunofluorescence.

**Nucleic Acid Manipulations**—Total RNA was prepared from the Me32a fibroblast cell strain as described previously (23). Five overlapping fragments spanning the 4.5-kb MNK coding region were generated by RT-PCR using Reverse Transcriptase AMV (Boehringer Mannheim) and recommended protocols. The primers employed for RT-PCR and subsequent nucleotide sequence analysis of the resultant RT-PCR products were designed from the previously published sequence of the MNK cDNA (4, 5). DNA sequencing was carried out using the Thermo Sequenase kit (Amersham Pharmacia Biotech). All other DNA manipulations were carried out using standard techniques (24).

**Western Blot Analysis**—Western blot analysis was carried out as described previously (25). The primary antibody consisted of sodium sulfate precipitated preparations of anti-MNK (21) or anti-WND4 antibodies directed against the N-termini of the human MNK and mouse WND proteins, respectively.

**Indirect Immunofluorescence**—Immunofluorescence analysis of cells was carried out essentially as described previously (21, 26). In general, for screening of G418-resistant clones, cells were cultured and stained in 96-well microtitre plates. Otherwise, cells were cultured on 13-mm glass coverslips for 48 h. Where appropriate, CuCl2 was added to the growth medium to a final concentration of 189 μM for 2–5 h, unless otherwise stated. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized in 0.1% Triton X-100 in PBS for 10 min, and quenched with 0.1 M ethanolamine. Nonspecific binding was blocked by incubation in 1% gelatin in PBS overnight, followed by incubation with the primary and secondary antibodies. The primary antibodies were the same as those employed for Western blot analysis.

**Copper Accumulation and Retention Studies**—Cells were cultured in basal medium and then transferred to BME supplemented with 2% FCS and trace amounts of 64Cu (as CuCl2, Australian Radiisotopes, Lucas Heights, NSW, Australia). The total copper concentration was 0.5 μM. For accumulation studies, cells were incubated for 24 h at 37°C, washed twice in cold BME, and harvested in 0.1% SDS. Following the Thermo Sequenase kit (Amersham Pharmacia Biotech). All other DNA manipulations were carried out using standard techniques (24). Western blot analysis was carried out as described previously (25). The primary antibody consisted of sodium sulfate precipitated preparations of anti-MNK (21) or anti-WND4 antibodies directed against the N-termini of the human MNK and mouse WND proteins, respectively.

**RESULTS**

**Determination of the Mutation within the ATP7A (MNK) Gene of Menkes Patient Fibroblast Strain Me32a—MNK protein was not detectable in Me32a cells.** MNK mRNA levels in these cells were too low to be detected by Northern blot analysis (4) but could be detected by RT-PCR. Therefore, RT-PCR was used to amplify the cDNA comprising the MNK coding region as five overlapping fragments. The nucleotide sequence of all but approximately 1 kb of the coding region was determined. A mutation was identified within the MNK cDNA sequence derived from the Me32a cells and comprised a deletion of four base pairs within exon 6. This exon encodes the region of the MNK protein between metal binding sites five and six. The mutation leads to the incorporation of a premature termination codon, 36 base pairs downstream from the four base deletion and would lead to the production of a truncated MNK protein, consistent with the failure to detect the MNK protein by Western blot analysis of Me32a cells.

**Expression of MNK and WND in Menkes Patient Fibroblasts**—The Me32a cell strain was immortalized by SV40 gene transfer and then designated Me32a-T22/2L. The 4.6-kb cDNAs encoding either human MNK (21, 25) or mouse WND4 proteins were cloned into a mammalian expression vector, pCMB77, which was derived by modifying the vector pCMB43 (25) to include an internal ribosome entry site. The resultant plasmids, pCMB117 (encoding MNK) and pCMB98 (encoding WND), were linearized and transfected into Me32a-T22/2L. Following G418 selection, colonies that expressed the proteins were identified by immunofluorescence analysis. Two independently derived clones that expressed MNK (A12-H9 and C3-C1) and two that expressed WND (WND-2 and WND-16) were chosen for further analysis.

To determine the MNK and WND protein expression levels in the clones, Western blot analysis was carried out using anti-MNK (21) or anti-WND4 antibodies, respectively, that were directed against the N-terminal region of the proteins. Clones A12-H9 and C3-C1 produced a protein band of the expected size (approximately 178 kDa), which was comparable in size with that produced by the normal human control fibroblasts (Fig. 1A). This MNK protein was not detected in the untransfected Me32a-T22/2L parental cell line or in this cell line transfected with the vector alone, Me32a-T22/2L (pCMB77). The lower molecular mass bands most likely represented degradation products because they were not consistently observed. In clones WND-2 and WND-16 and in the mouse liver sample, the anti-WND antibodies detected a band of approximately 165 kDa (Fig. 1B), which is the expected size of the WND protein (28). This band was not detected in the parental cell line, Me32a-T22/2L (pCMB77), or in the control fibroblasts. Note that the anti-WND antibodies were specific for mouse WND and would not cross-react with any endogenously expressed human WND in these cells. However, human WND mRNA could not be detected in the control fibroblasts by Northern blot analysis using a human WND cDNA probe (data not shown).

**Copper Accumulation and Retention Studies**—To determine whether the expressed proteins could correct the copper transport abnormality of the Menkes fibroblasts, copper transport studies were carried out. Following a 24-h period in medium containing trace amounts of 64Cu, the parental Me32a-T22/2L and Me32a-T22/2L (pCMB77) cell lines accumulated approximately 2-fold more copper than the control fibroblasts GM847 and GM2069 SV9 (Table I). The MNK-expressing clones showed a decreased accumulation of copper to levels that were significantly lower than those of both the Menkes and the control cell lines. Copper accumulation by the WND-expressing
clones. Whole cell protein extracts were prepared from the non-Menkes control for the WND antibodies. Approximately 30 WND clones WND-2 and WND-16, and mouse liver tissue as a positive T22/2L, Me32a-T22/2L (pCMB77), MNK clones A12-H9 and C3-C1, human fibroblast cell lines (GM639, GM847, and GM2069 SV9), Me32a- was consistent with a TGN location as previously reported for MNK in the control fibroblast line, GM847 (Fig. 2). This result was consistent with the previous studies of MNK in CHO cells (21, 26).

In the WND-expressing clones, WND also was located in the perinuclear region consistent with previous studies indicating a TGN location for WND (28, 31) (Fig. 3). However, in contrast to MNK, WND did not relocalize with elevated copper levels but remained predominantly within the perinuclear region (Fig. 3), even after prolonged (24 h) incubation in elevated copper levels. This result was not likely to be due to an abnormality of the cDNA-derived WND protein because WND expressed from the same expression plasmid in CHO cells was redistributed to a cytoplasmic compartment with elevated copper conditions as previously observed with endogenously expressed WND in HepG2 cells (28). Consistent with the Western blot data, the antibodies did not detect any WND protein in the normal control fibroblasts (GM847) or in the parental Menkes fibroblasts.

**FIG. 1. Western blot analysis of MNK- and WND-expressing clones.** Whole cell protein extracts were prepared from the non-Menkes human fibroblast cell lines (GM639, GM847, and GM2069 SV9), Me32a- T22/2L, Me32a-T22/2L (pCMB77), MNK clones A12-H9 and C3-C1, WND clones WND-2 and WND-16, and mouse liver tissue as a positive control for the WND antibodies. Approximately 30 μg of total cell protein was fractionated by SDS-polyacrylamide gel electrophoresis (7.5% gel) and transferred to nitrocellulose filters. The filters were probed with either anti-MNK antibodies directed against the MNK N terminus (21) (A) or anti-WND antibodies directed against the mouse WND N terminus (B). The secondary antibody consisted of horseradish peroxidase-conjugated sheep anti-rabbit IgG (AMRAD Biotech). Protein detection was carried out using the Chemiluminescent POD substrate (Boehringer Mannheim). The positions of the molecular mass markers (Bio-Rad) are indicated on the left in kDa.

clone, WND-16, was reduced compared with the Menkes fibroblasts but was comparable with the control fibroblasts (Table I). After a further 24 h in medium without 64Cu, the amount of 64Cu that was retained within the cells was measured as an indirect measure of copper efflux. The results showed that the MNK and WND clones and the control fibroblasts retained substantially less copper than the Me32a-T22/2L and Me32a-T22/2L (pCMB77) cell lines (Table I). In the WND-expressing clones, the MNK protein was located in the perinuclear region (Fig. 2), similar to the endogenously expressed MNK in the control fibroblast line, GM847 (Fig. 2). This result was consistent with a TGN location as previously reported for MNK (21, 26, 29, 30). There was no MNK protein detected in the parental Me32a-T22/2L fibroblasts. In the presence of elevated extracellular copper, MNK in both the normal control (GM847) and in the transfected fibroblasts was redistributed to the cytoplasm and plasma membrane (Fig. 2), again consistent with previous studies of MNK in CHO cells (21, 26).

DISCUSSION

A definitive biochemical diagnosis for Menkes’ disease is based on increased accumulation and retention of copper in cultured cells from Menkes patients, which are indirect indicators of defective copper efflux (13, 16–19). The cell strain Me32a was obtained from a patient with classical Menkes’ disease and showed a copper accumulation and retention phenotype that was typical of the disease. In this study the cDNA-derived MNK protein was able to restore copper transport activity by this cell line. This report represents the first demonstration that the expression of this protein alone was able to correct the copper accumulation and retention defect of Menkes fibroblasts. WND did not appear to be endogenously expressed in fibroblasts, but the expression of this protein from a cDNA construct also led to correction of the copper transport defect of these cells, although there were differences in the kinetics of copper transport compared with MNK.

There was an obvious difference between the parental Menkes fibroblasts and the MNK- and WND-expressing clones in the amount of copper that had accumulated over 24 h and in the amount that was subsequently retained during the 24-h chase period. For the MNK clones, the reduction in copper accumulation to levels below normal may have been due to the expression of the cDNA-derived MNK above the normal level, as indicated by immunofluorescence (Fig. 2). Increased expression of MNK was shown to result in enhanced copper efflux (32). The difference in the copper accumulation phenotype between the MNK and WND clones may relate to a greater efficiency of copper efflux by MNK, which would result in a significantly reduced pool of copper in the MNK clones by the end of the accumulation period. The apparently lower retention of copper by WND-16, compared with the MNK clones, then may be explained by a higher proportion of copper in these cells that was available for efflux during the 24-h efflux period. The copper accumulation and retention properties of the transfected clones were consistent with preliminary experiments that assessed the copper resistance of these clones. When cells were exposed to a medium copper concentration of 125 μM for 48 h, cell viability of the untransfected and vector-transfected Menkes fibroblasts was 3–4 fold lower than that of the normal control fibroblasts and the Menkes fibroblasts that expressed MNK and WND (data not shown).

The current evidence suggests that in basal copper levels, MNK is located in the TGN (26, 29, 30), where presumably it transports copper into the TGN lumen for incorporation into
Lysyl oxidase and other secreted cuproenzymes. Recent studies with MNK-overexpressing CHO cells also suggested that MNK constitutively recycles between the TGN and the plasma membrane from where copper is effluxed from the cell (26). However, with increased intra- and extracellular copper levels, the steady state distribution of MNK shifts toward the plasma membrane, presumably to increase the efficiency of removal of the excess copper from the cell (26). The association of cDNA-derived MNK with vesicles within the cell and with the TGN and plasma membrane was confirmed at an ultrastructural level in CHO cells (21, 26). Using the same MNK cDNA construct, the results obtained in this study demonstrating MNK location and its response to elevated extracellular copper levels in Menkes fibroblasts were consistent with previous data obtained with CHO cells. The greater efficiency of copper efflux resulting from MNK expression compared with efflux resulting from WND expression in the same cell type may relate to the ability of MNK to recycle between the TGN and plasma membrane in basal medium, as demonstrated in CHO cells (26). This study showed that, albeit in elevated copper conditions, MNK in the Menkes fibroblasts was able to localize to the plasma membrane, which suggested that it retained similar properties in CHO cells and in fibroblasts. Therefore, based on the copper transport studies and intracellular distribution in the absence and presence of elevated copper, we concluded that the cDNA-expressed Menkes protein was fully functional in the absence of copper.

### Table I

| Cell Line           | Copper accumulation (mean ± S.E.) | Copper retention (%)         |
|---------------------|----------------------------------|------------------------------|
|                      | pmol/mg protein                  |                              |
| Menkes - Me32a-T22/2L| 0.99 ± 0.044                     | 73 (70, 75)                  |
| Menkes - Me32a-T22/2L(pCMB77)| 1.08 ± 0.046             | 75 (78, 71)                  |
| Normal - GM847       | 0.62 ± 0.002                     | 44 (39, 48)                  |
| Normal - GM2069 SV9  | 0.43 ± 0.018                     | 31 (30, 31)                  |
| Transfected - A12-H9 | 0.07 ± 0.002                     | 37 (37, 37)                  |
| Transfected - C3-C1  | 0.06 ± 0.002                     | 44 (39, 48)                  |
| Transfected - WND-16 | 0.38 ± 0.018                     | 23 (24, 21)                  |

a Values for accumulation represent the amount of copper accumulated in cells after 24 h in 64Cu-containing medium, expressed as the means ± S.E. of four independent measurements.

b Values for retention represent the amount of copper remaining in cells after a further 24 h in medium lacking 64Cu, expressed as percentages of the amount of copper accumulated in cells after 24 h. Values represent the mean of the two independent measurements shown in parentheses.

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**Fig. 2.** Subcellular localization and effect of copper on the intracellular distribution of MNK in Menkes fibroblasts. GM847, Me32a-T22/2L, A12-H9, and C3-C1 were cultured for 48 h in basal medium and then incubated in the absence (−Cu) or in the presence of 189 μM copper (as CuCl₂) (+Cu) for 2–3 h at 37 °C. The cells were fixed, and MNK was detected with a sodium sulfate-precipitated preparation of anti-MNK antibodies, followed by fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG antibodies.
Menkes fibroblasts, and alone could correct the copper efflux defect of these cells.

The WND protein also restored copper efflux by the Menkes cells but did not display the copper-induced redistribution pattern exhibited by MNK. Despite an apparent lack of endogenously expressed WND in fibroblasts, the cDNA-expressed WND protein had a perinuclear location that was consistent with localization to the TGN, as previously demonstrated in human hepatoma cells (28, 31, 33) and LEC rat hepatocytes (34, 35). The presence of WND at the TGN is consistent with a role for this protein in the delivery of copper to ceruloplasmin (34, 35). A smaller 140-kDa form of WND has been localized to a cytosolic compartment (31, 36), which was identified as mitochondria (33), and was consistent with WND-specific cytosolic staining in HepG2 cells noted by Hung et al. (28). This finding suggested an unexpected role for WND in the delivery of copper to proteins involved in energy-generating processes within the cell (33). In the present study only the TGN-localized 165-kDa form of WND was identified. There have been conflicting reports on the effect of copper on the intracellular distribution of WND in Menkes fibroblasts, with one study reporting an absence of copper-induced redistribution of WND (31), whereas a different group reported copper-induced movement of WND to a cytoplasmic vesicular compartment but not to the plasma membrane as observed with MNK (28). However, copper ATPase activity has been detected in the plasma membrane fraction of rat hepatocytes (37), and the presence of WND at the plasma membrane of LEC rat hepatocytes was reported following in vivo experiments that involved direct injection of a WND expression plasmid into rat liver (35). These results are consistent with a role for WND in the secretion of copper into bile and therefore with its presence at the biliary canaliculus membrane, which is a polarized surface. Therefore, it is likely that the inability to detect WND at the plasma membrane of HepG2 cells may be due to the lack of polarization of these cells in culture.

In this study, the fact that WND may not be normally expressed in fibroblasts and/or the lack of polarization of these cells may account for the observed failure of copper to induce the relocalization of WND to the plasma membrane in the Menkes fibroblasts. However, because the WND-expressing fibroblasts demonstrated an enhanced capacity for copper efflux, it is possible that a low basal level of WND recycling between the TGN and either the plasma membrane or another cellular compartment may have been occurring, which was sufficient to reduce the copper accumulation and restore copper efflux by these cells but was not sufficient to enable detection by immunofluorescence. Alternatively, copper may be transported by WND into the TGN lumen and eliminated from the cell in a protein-bound form via the secretory pathway. If WND is not normally expressed in fibroblasts, then the apparent failure of WND to respond to elevated copper may be due to a lack of either the appropriate trafficking components that are required to specifically interact with WND or intracellular targets for WND-containing vesicles.

The Menkes and Wilson proteins have similar structures and

![Figure 3. Subcellular localization and effect of copper on the intracellular distribution of WND in Menkes fibroblasts. GM847, Me32a-T22/2L, WND-2, and WND-16 were cultured for 48 h in basal medium and then incubated in the absence (-Cu) or in the presence of 189 μM copper (as CuCl₂) (+Cu) for 2–3 h at 37 °C. The cells were fixed, and WND was detected with a sodium sulfate-precipitated preparation of anti-WND antibodies, followed by fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG antibodies.](image-url)
perform similar functions, although in different cell types. The data presented in this study were consistent with a common ability of both MNK and WND to translocate copper across cellular membranes and represents the first report of the expression and function of these two proteins in the same cell type. Under these conditions, WND activity could at least partially substitute for MNK function, although the pathway for elimination of copper from the cell may differ for the two proteins. Although the relocation studies were carried out using copper concentrations that were significantly higher than those employed for the copper accumulation and retention studies, the difference in MNK and WND distribution in elevated copper may reflect differences in the way that the two proteins transport copper across the cell.

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