A copper treatable Menkes disease mutation associated with defective trafficking of a functional Menkes copper ATPase

B-E Kim, K Smith, M J Petris

J Med Genet 2003;40:290–295

Copper dependency in humans is most dramatically illustrated in Menkes disease, an X linked recessive copper deficiency disorder that is generally lethal in early childhood.^{1,2} Menkes disease is caused by mutations in a transmembrane copper transporting P type ATPase, MNK (or ATP7A), which is expressed in virtually all non-hepatic tissues.^{3–5} Studies using cultured cells suggest that MNK is located in the trans-Golgi network (TGN), where it transports copper to copper dependent enzymes synthesised within secretory compartments.^{6–8} In addition to this biosynthetic role, MNK functions in the efflux of excess copper from cells via a process of copper stimulated trafficking to the plasma membrane.^{6,9} Copper export via MNK from intestinal enterocytes is essential for supplying the blood with dietary copper. Similarly, MNK mediated copper export from the capillary endothelium of the blood brain barrier is thought to supply copper to the central nervous system. In Menkes patients, these processes are defective resulting in a range of symptoms attributable to deficiencies in copper dependent metabolism. These include neurological degeneration, mental retardation, seizures, arterial and bone abnormalities, hypothermia, and hypopigmentation.² Classical Menkes disease rapidly progresses and is generally lethal during early childhood, although milder variants of the disease exist.^{10–11}

The treatment of Menkes disease involves parenteral injections of copper-histidine, which in the most successful cases reduces neurological defects and prolongs life expectancy.^{2,12–13} This copper replacement therapy bypasses the intestinal blockage of dietary copper absorption and increases circulating copper levels. However, to prevent the onset of neurological symptoms in Menkes patients, copper must be delivered across the endothelial cells of the blood brain barrier to supply copper to the central nervous system. Within the central nervous system, copper transport into secretory compartments of neurones and other cells to supply copper to cuproenzymes, such as dopamine β hydroxylase and peptidylglycine α amidating mono-oxygenase, may also be important for the successful treatment of Menkes disease. The clinical outcome of copper replacement therapy for the small number of reported human cases ranges from poor¹⁴ to favourable,^{15–16} with no definitive biochemical explanation for this variability. To begin to understand the contribution of the Menkes genotype to the success of copper replacement therapy, we characterised the biochemical and cell biological defect associated with a MNK mutation previously identified in a successfully treated patient.¹⁵ This mutation involves a splice site mutation resulting in the deletion of exon 8, which encodes a small region between the sixth copper binding site and the first membrane spanning domain of the MNK protein. In this study, we assessed the copper induced trafficking and copper transport function of this exon 8 skipped MNK protein.

Key points

- A Menkes disease mutation resulting in the deletion of exon 8 in the MNK gene was previously identified in a Menkes patient successfully treated with copper replacement therapy. We investigated the effect of this mutation on the trafficking and copper transport function of the mutant MNK protein (MNK/III.6).
- The MNK/III.6 protein localised correctly to the TGN and was able to transport copper to tyrosinase, a copper dependent enzyme that is synthesised within secretory compartments. However, in cells exposed to increased copper, the MNK mutant protein failed to traffic to the plasma membrane. This trafficking defect was also confirmed in primary cultured cells from an affected cousin of the successfully treated patient with the same mutation.
- This mutation represents the first trafficking defective Menkes disease mutation that retains copper transport function, thereby showing that trafficking and transport functions of the MNK ATPase can be uncoupled.
- We conclude that certain Menkes disease mutations that inhibit copper induced trafficking of an otherwise functional copper transporter may be particularly responsive to copper replacement therapy.

MATERIALS AND METHODS

Reagents and cell culture

All chemicals were purchased from Sigma. The immortalised Menkes fibroblast cell line, Me32a, lacks detectable MNK protein owing to an early frameshift mutation in MNK, as described previously.¹⁷ The primary Menkes patient fibroblast cell line, GM13672, and control primary fibroblasts, GM00302 and GM00970, were obtained from the Coriell Genetic Cell Repository. The GM13672 cell line was originally derived from the affected cousin of patient III.6 (patient 1433 in Das *et al.*¹⁸ All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 100 U/ml penicillin and streptomycin in a 5% CO₂, 37°C incubator.

Plasmids and transfections

The MNK/III.6 mutation, which replaces the exon 8 encoded amino acids with an Ile-Arg dipeptide, was generated by PCR, verified by DNA sequencing, and reconstituted into the full length MNK construct described previously.¹⁹ Plasmid constructs bearing the wild type MNK or MNK/III.6 mutant cDNA were transiently transfected into Me32a cells using lipofectAMINE 2000 (Gibco BRL), according to the manufacturer's instructions. Plasmid constructs expressing the MNK mutations D1044E and C1000R have been described

previously.^{8,20} Transfection efficiencies were typically 30% of Me32a cells and images shown are representative of the population of transfected cells.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as previously described,²¹ using affinity purified MNK antibodies, and detected using Alexa594 anti-sheep secondary antibodies (Molecular Probes).

Tyrosinase assay

Tyrosinase activity was determined using a colorimetric assay based on the enzyme's DOPA oxidase activity, as we have previously described.^{8,22} Me32a cells were pre-grown overnight in 25 cm² flasks and LipofectAMINE 2000 was used to transiently transfect these cells with 2 µg of the tyrosinase plasmid, pcTYR,²³ together with 2 µg of the various MNK expression plasmids indicated in the figure legends. After culturing for 48 hours, cells were harvested by scraping in phosphate buffered saline, pelleted, and sonicated in a buffer containing 2% SDS, 62.5 mmol/l Tris-Cl (pH 6.8), protease inhibitor cocktail (Roche), 1 mmol/l ascorbate, and 1 mmol/l bathocuproine disulphonate to chelate free copper. Cell lysates (20 µg) were fractionated by non-reducing 7.5% SDS-PAGE and tyrosinase activity was colorimetrically determined by incubating gels for 15 minutes at 37°C in 10 mmol/l phosphate buffer (pH 6.8) containing 1.5 mmol/l L-3,4-dihydroxyphenylalanine and 4 mmol/l 3-methyl-2-benzothiazolinone hydrazone (Sigma). Immunoblotting experiments were performed using either sheep anti-MNK antibodies or goat anti-tyrosinase antibodies (Santa Cruz Biotech), using an enhanced chemiluminescence detection kit (Roche). Tyrosinase experiments were performed four times with similar results to the gel shown.

Crude membrane preparations

Primary fibroblasts were grown to confluency in 75 cm² flasks, scraped into PBS, and pelleted at 1000 × *g*. Cells were resuspended in 400 µl 62.5 mmol/l Tris-Cl (pH 6.8), 1 mmol/l EDTA, and a protease inhibitor cocktail (Roche), and disrupted by sonication. Cells were centrifuged for two minutes at 10 000 × *g* and the supernatant was collected and recentrifuged for 30 minutes at 100 000 × *g* to pellet the total crude membranes. The membranes were resuspended in 100 µl of a buffer containing 2% SDS, 62.5 mmol/l Tris-Cl (pH 6.8), 1 mmol/l EDTA, and a protease inhibitor cocktail, and 20 µg samples were separated using 4-20% SDS-PAGE, transferred to nitrocellulose membranes, and MNK protein was detected by enhanced chemiluminescence as described above.

RESULTS

A previous study reported the successful treatment of a Menkes patient, designated III.6, by copper replacement therapy.¹⁵ Patient III.6 was born to a family with an older half brother and first cousin previously diagnosed with Menkes disease. On day 8 of life, parenteral copper histidine was administered to patient III.6 after a positive diagnosis of Menkes disease. During infancy, patient III.6 showed normal head growth, brain myelination, and age appropriate neurodevelopment. In contrast, the affected half brother and first cousin with the same mutation as patient III.6, who were not diagnosed and treated with copper replacement therapy, showed typical symptoms of classical Menkes disease, including arrested head growth, cerebral atrophy, delayed myelination, and abnormal neurodevelopment.^{15,18}

The mutation common to all three Menkes patients of this family was a tandem duplication of five nucleotides at the splice acceptor site preceding exon 9 of the 23 exon *MNK* gene (IVS8, AS, dup5).^{15,18} This resulted in two mutant transcripts in all three patients in this family. The first mutant transcript has an altered reading frame that terminates four bases into

Figure 1 Schematic diagram of the predicted MNK protein generated from the in frame exon 8 skipped transcript of Menkes patient III.6. The filled squares indicate positions of the six copper binding sites at the amino terminal region of MNK containing the C-X-X-C motif. The eight transmembrane regions are shown along with the location of amino acids mutated in this study (C1000 and D1044). The region spanning the sixth copper binding site to the first transmembrane region is enlarged showing the exon 8 encoded sequence between serine-624 and glutamine-649 that is deleted in the in frame transcript of Menkes patient III.6 and replaced with 624IR.

exon 9, resulting in a predicted protein which lacks all membrane spanning regions and ATPase catalytic domains, and therefore predicted to have no copper transport function. However, the second transcript contained the 5 bp insertion as well as a deletion of exon 8. These combined alterations maintain the open reading frame and result in the deletion of amino acids 624-649 encoded by exon 8 with the concomitant insertion of two novel residues Ile-Arg.¹⁵ We have referred to this mutant protein as MNK/III.6 (fig 1). To begin to understand the defects associated with a copper responsive Menkes mutation at the intracellular level, we characterised the biochemical and cell biological function of the MNK/III.6 protein.

In vitro mutagenesis was used to generate the MNK/III.6 mutant open reading frame in a plasmid bearing the MNK cDNA. Both wild type MNK (WtMNK) and the MNK/III.6 mutant protein were expressed in the immortalised fibroblast cell line, Me32a. This cell line was derived from a Menkes patient and does not express endogenous MNK protein.¹⁷ Me32a cell lines were transfected with the MNK/III.6 plasmid and clonal cell lines were selected for resistance to G418. However, despite an initially high proportion of cells expressing the MNK/III.6 protein, this expression was rapidly lost during clonal expansion. A similar instability has been reported in cell clones expressing other MNK proteins with mutations in this region.²⁴ To circumvent the loss of MNK/III.6 expression associated with passaging of cells, we assessed the intracellular location of the MNK/III.6 protein in transiently transfected cells, as we have done previously.²¹ Immunofluorescence microscopy showed that the WtMNK protein was located in the perinuclear region of cells (fig 2A), consistent with its location in the TGN.^{6,7} There was no signal detected in Me32a cells stably transfected with the empty vector (data not shown). The MNK/III.6 protein was detected in the perinuclear region of cells suggesting the protein was correctly located in the TGN (fig 2B). Further evidence of a TGN location of the MNK/III.6 protein was the contraction of the perinuclear signal in cells treated with brefeldin A (fig 2D), a drug which causes contraction of the TGN to the microtubule organising centre.²⁵ This contraction was also observed for the WtMNK protein in brefeldin A treated cells (fig 2C), as we have shown previously.⁶

We then investigated whether the MNK/III.6 protein was able to traffic from the TGN to the plasma membrane in cells

Figure 2 Immunofluorescence microscopy analysis of the intracellular location of the MNK/III.6 protein in transfected cells. Me32a cells expressing WtMNK or MNK/III.6 protein were either untreated (A, B), treated for two hours in media containing 5 $\mu\text{mol/l}$ brefeldin A (C, D), or treated for two hours with 200 $\mu\text{mol/l}$ copper (E, F). Cells were probed with affinity purified MNK antibodies (1:1500) followed by a fluorescent Alexa 594 labelled secondary antibody (1:1000), and viewed using 60 $\times$ oil objective. Note the perinuclear location of the MNK/III.6 protein in untreated cells and the contraction of this signal in brefeldin A treated cells, similar to the WtMNK protein. The copper induced trafficking of the WtMNK protein is not apparent for the MNK/III.6 protein.

exposed to media containing 200 $\mu\text{mol/l}$ copper. In copper treated cells, the WtMNK protein relocated from the TGN to the plasma membrane as evident by the dispersed staining that was associated with the cell periphery (fig 2E). However, in copper treated cells the perinuclear location of the MNK/III.6 protein was unchanged (fig 2F). The exposure of cells to higher copper levels for longer times did not alter the TGN location of the MNK/III.6 protein (data not shown). These observations suggested that the MNK/III.6 mutation inhibits copper induced trafficking of the MNK protein.

To explore whether the MNK/III.6 mutant protein retains copper transport function, we assessed its ability to activate tyrosinase. Tyrosinase is a copper dependent enzyme with a single membrane spanning region and a catalytic copper binding domain located on the luminal side of secretory compartments.²³ We have recently shown that the activity of tyrosinase requires copper to be transported by the MNK protein from the cytoplasm into secretory compartments.⁸ We confirmed these findings in this study by showing tyrosinase activation in Me32a cells co-transfected with both the tyrosinase and wild type MNK constructs (fig 3, lane 2). As expected, the control vector (lacking MNK) and the MNK mutant, D1044E, which inhibits copper transport by preventing catalytic phosphorylation of MNK,²⁶ failed to activate tyrosinase (fig 3, lanes 1 and 4). Significantly, the MNK/III.6 protein was able to activate tyrosinase to similar levels as the WtMNK (fig 3, lane 3). We have recently shown another Menkes disease mutation, C1000R, prevents copper induced trafficking of MNK from the TGN to the plasma membrane.²⁰ The C1000R mutation affects the highly conserved CPC motif in the sixth membrane of MNK, and is highly conserved in other copper transporting ATPases.²⁷ In contrast to the MNK/III.6 protein, the C1000R

Figure 3 Tyrosinase activation by the MNK/III.6 protein. Me32a cells co-transfected with the tyrosinase expression plasmid, pcTYR, and either the vector alone (lane 1), or the MNK expression plasmids for WtMNK (lane 2), MNK/III.6 (lane 3), MNK-D1044E (lane 4), or MNK-C1000R (lane 5). The upper panel shows the bands produced by the oxidation of L-DOPA by tyrosinase in transfected cells. The middle panel is an immunoblot showing the corresponding levels of each MNK mutant protein in the transfected cells detected using affinity purified MNK antibodies. The lower panel is an immunoblot showing the corresponding levels of tyrosinase protein in each sample.

mutant failed to activate tyrosinase (fig 3, lane 5). These findings suggest that while both the C1000R and MNK/III.6 mutations inhibit copper induced trafficking, only the MNK/III.6 protein retains copper transport function.

A previous study showed that levels of the exon 8 skipped transcript were reduced in primary fibroblasts from all three affected members of the MNK/III.6 patient family. Thus, it was expected that these cells would possess reduced levels of the MNK/III.6 protein relative to levels of the wild type MNK protein in normal fibroblasts.^{15, 18} Furthermore, our analysis of the recombinant MNK/III.6 mutant protein predicts that the endogenous MNK/III.6 protein in these patient cells would be located in the TGN, but unable to relocate when cells were exposed to increased copper. To test these hypotheses, we investigated the levels and intracellular distribution of the endogenous MNK protein in primary cultured fibroblasts derived from the affected cousin of patient III.6, known as patient 1433.¹⁸ These patient cells, designated GM13672, were obtained from the Coriell Genetic Cell Repository along with two normal unrelated control lines, GM00970 and GM00302. Initially we were unsuccessful in detecting MNK protein using western blots of total cell lysates derived from GM13672 cells. This was potentially because the levels of the MNK/III.6 protein were below the limits of detection with our MNK antibody. To enrich MNK protein levels in our cell extracts, we purified crude membranes from the GM13672 and normal control cell lines. Immunoblot analysis of these membrane preparations with the MNK antibody resulted in the detection of MNK protein in GM13672 cells at levels which were markedly lower than MNK protein levels in both the GM00970 and GM00302 control cell lines (fig 4A). These findings suggest that the exon 8 skipped transcript in these patient cells resulted in the expression of MNK/III.6 protein, albeit at reduced levels relative to normal control cells.

Figure 4 Reduced levels and defective copper induced trafficking of the MNK protein in primary fibroblasts with the MNK/III.6 mutation. (A) Immunoblot detection of MNK protein levels in membrane preparations from the primary fibroblast cell lines. The GM13672 cell line was derived from Menkes patient 1433, an affected cousin of patient III.6 with the same mutation, and the GM00302 and GM00970 cells are normal primary fibroblasts. (B-G) Immunofluorescence microscopy with affinity purified MNK antibodies to detect the location of MNK protein in the cultured primary cells exposed to basal media or for two hours in media containing 200 μmol/l copper (+Cu).

We then investigated the intracellular location of the endogenous MNK protein in GM13672 cells by immunofluorescence microscopy. A weak perinuclear signal was detected in GM13672 cells cultured in basal media (fig 4B) and the location of this signal did not change in cells exposed to increased copper (fig 4C). In both the control cell lines, GM00302 and GM00970 cultured in basal medium, the perinuclear signal was stronger than in the GM13672 cells (fig 4D, F), consistent with the raised level of MNK protein in these normal cells. The expected copper induced trafficking of MNK from the TGN was observed in both these control cell lines, as evidenced by the loss of perinuclear labelling and the appearance of dispersed staining extending to the cell periphery (fig 4E, G). These findings using cultured cells derived from the affected cousin of patient III.6 confirmed our earlier results with transfected cells and suggested that the deletion

of exon 8 in the MNK/III.6 protein prevented the copper induced trafficking of MNK.

DISCUSSION

This study was aimed at characterising the cell biological and biochemical defects associated with a Menkes mutation in a patient who was successfully treated with copper replacement therapy. Immunocytochemical studies in transfected cells showed that the MNK/III.6 protein was localised in the TGN but failed to undergo copper induced trafficking. These findings were confirmed in primary fibroblasts derived from an affected cousin of patient III.6 with the same mutation. Western blot analysis of these fibroblast cells indicated reduced expression of the MNK/III.6 protein, consistent with previous findings of lower levels of the exon 8 skipped MNK transcript in these cells relative to levels of the wild type MNK transcript in normal fibroblasts.¹⁵ An important finding of this study was that the MNK/III.6 protein was able to activate tyrosinase and was therefore a functional copper transporter. This suggests that the ability of MNK to undergo trafficking is not essential for its function in copper transport to cuproenzymes in the secretory pathway.

We have recently shown that the copper induced trafficking of MNK is coupled with its catalytic activity by showing that mutations that prevent formation of the phosphorylated catalytic intermediate also prevent copper induced trafficking.²⁰ Our analysis of the MNK/III.6 protein enlarges on this model by indicating that mutations that inhibit copper induced trafficking of MNK do not necessarily abolish its catalytic activity. Hence, the MNK/III.6 mutation shows that the copper induced trafficking and copper transport functions of MNK can be uncoupled. In contrast to the MNK/III.6 mutation, the C1000R mutation in the conserved CPC motif prevented copper transport to tyrosinase. The C1000R mutant, together with the D1044E mutation, is likely to represent a class of MNK mutant that prevents copper induced trafficking owing to inhibited catalytic turnover.²⁰ Although the copper induced trafficking of MNK requires catalysis of the protein, the mechanism involved in the sorting of MNK into exocytic vesicles is unknown. The six copper binding sites at the amino terminal region of MNK are required for catalytic function under low copper levels,²⁶ and the fifth or sixth copper binding site must be intact for the trafficking response under increased copper levels.²⁸ Our analysis of the MNK/III.6 protein, which lacks the 26 amino acids between the sixth copper binding site and the first transmembrane domain, suggests this mutation may interfere with the role of the fifth and sixth copper binding sites in triggering exocytic MNK trafficking.

How does our analysis of defects associated with the MNK/III.6 mutation explain the pathogenesis of Menkes disease in this family? Lower levels of MNK/III.6 protein caused by the splicing defect, together with defective copper induced trafficking to the plasma membrane, probably reduced the export of dietary copper from intestinal enterocytes into circulation. Furthermore, copper transport to the brain was also likely to be impaired since the two untreated Menkes patients in this family developed severe neurological symptoms and cerebral and cerebellar atrophy.¹⁵ Previous studies showed that cultured fibroblasts from all three affected Menkes patients in this family expressed similar levels of the exon 8 skipped transcript, and copper retention by these cells was similarly increased relative to normal controls.¹⁵ Therefore, it is probable that patient III.6 would have developed the severe neurological symptoms experienced by his older relatives if copper replacement therapy had not been administered. The extent to which decreased MNK/III.6 protein with normal or partial copper transport function and the defective trafficking of this protein contributed to the disease remains to be clarified, and indeed whether normal levels of MNK/III.6 would have resulted in a disease phenotype.

How does our analysis of the defects associated with the MNK/III.6 mutation explain the positive response to copper treatment in patient III.6? As with other inherited metabolic disorders with considerable genetic and phenotypic variability, the greatest potential for successful treatment is for those mutations that result in residual activity of the affected protein. Genotypic studies have indicated that a correlation exists between the clinical severity of Menkes disease and the type of mutation.^{18, 29-31} Null mutations involving deletions and early frameshifts have been identified only in severely affected patients with classical Menkes disease, whereas milder forms of Menkes disease have been associated exclusively with splicing mutations or missense mutations where the possibility exists for the expression of MNK protein with residual copper transport function.³⁰ As with the correlation between genotype and disease severity, it is highly probable that the response to copper therapy is also closely correlated with genotype, as is the case with treatment response for other inherited metabolic disorders. A well studied example is the inherited disorder phenylketonuria, in which the activity of the affected enzyme, phenylalanine hydroxylase, varies with mutation type, and patients display a broad range of tolerance to phenylalanine and response to dietary restriction of this amino acid.³² In the successfully treated patient III.6, the ability of the MNK/III.6 protein to transport copper from the cytoplasm into the TGN may have contributed to the favourable neurological response to copper replacement therapy. In this scenario, the increased serum copper levels following copper injections may have provided enough copper to the MNK/III.6 protein to allow clinically meaningful copper delivery to the brain despite the inability of this protein to undergo copper induced trafficking from the TGN. This could have occurred via copper transport by the MNK/III.6 protein into the TGN within the capillary endothelial cells of the blood brain barrier, with subsequent vesicular transport via the secretory pathway to the plasma membrane, permitting excretion of copper into the central nervous system. It is conceivable that some patients with complete deletions of MNK may respond to copper replacement therapy if alternative copper transport pathways to the brain were available. However, the MNK/III.6 patient mutation is likely to represent one of several classes of mutation involving some functional MNK protein where copper replacement therapy is particularly efficacious. Other examples include missense mutations, small in frame deletions, promoter mutations, and splice site mutations that permit some proper splicing. It is clear that further analysis of mutations in copper treated patients at the clinical, biochemical, and cell biological levels are required to clarify the degree to which the Menkes genotype influences the efficacy of early copper replacement therapy. Ultimately the effective treatment of Menkes disease regardless of mutation type may require the development of copper complexes that are able to cross the blood brain barrier and/or membranes of secretory compartments, with properties that allow donation of this copper to essential cuproenzymes.

ACKNOWLEDGEMENTS

We are grateful to Drs David Eide and Elizabeth Rogers for helpful discussions and critical reading of the manuscript. We thank the clinicians and scientists for providing patient cell lines to the Coriell Genetic Cell Repository. This work was funded by the National Institutes of Health (grant DK59893).

Authors' affiliations

B-E Kim, K Smith, M J Petris, Department of Nutritional Sciences, University of Missouri, Columbia, MO 65211, USA

Correspondence to: Dr M J Petris, Department of Nutritional Sciences, 217 Gwynn Hall, University of Missouri-Columbia, Columbia, MO 65211, USA; PetrisM@missouri.edu

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