Molecular basis of the brindled mouse mutant (Mo\textsuperscript{bn}): a murine model of Menkes disease

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INTRODUCTION

The brindled mouse mutant (Mo\textsuperscript{bn}) is the closest animal model of the human genetic copper deficiency, Menkes disease, which is presumed to be due to a mutation at the X-linked mottled locus (Mo). The mutant mice are hypopigmented and die at around 15 days after birth, but can be saved by treatment with copper before the 10th postnatal day. Menkes disease has been shown to be due to mutations of the gene ATP7A which encodes P-type ATPase (referred to here as MNK). MNK is likely to function in copper efflux from cells, but the full range of its biological activity is not fully understood. The nature of the mutation in the brindled mouse is of importance in our understanding of the role of MNK and for devising treatment strategies for Menkes disease. Here we show that the brindled mouse has a deletion of two amino acids in a highly conserved, but functionally uncharacterized, region of Mnk. Comparison with the Ca ATPases suggests this region may be involved in conformational changes associated with the E1/E2 transition fundamental to the action of P-type ATPases. We also describe the first Western blot data for Mnk in tissues, and these show normal levels of Mnk in mutant and brindled kidneys but none in liver. In the kidney, immunohistochemistry demonstrated Mnk in the proximal and distal tubules, the distribution is identical in mutant and normal. This distribution is consistent with Mnk being involved in copper resorption from the urine.

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INTRODUCTION

Menkes disease is an X-linked copper deficiency disorder resulting in death in early childhood. Affected boys have severe neurological problems, abnormal hair, connective tissue defects and suffer from hypothermia due to the low activity of various copper-dependent enzymes (1). The gene affected in Menkes disease (MNK) has been isolated by positional cloning (2–4) and encodes a protein termed MNK (or ATP7A) which is a member of the family of cation-transporting P-type ATPases (4). MNK is thought to mediate copper efflux from cells (5), but its full biological role is not understood. A complete understanding of the role of MNK in copper transport will require analysis of the expression and distribution in animal tissues. MNK mRNA is found in many human (2,4) and mouse (6) organs, but the liver contains little MNK mRNA, instead expressing the closely related copper transporter, WND, which is defective in Wilson disease (7–9).

The mottled mice (Mo) are an interesting series of mutant mice which are due to allelic mutations of the murine homologue of the Menkes gene, Mnk (Atp7a). The phenotype of these mice is quite varied, including prenatal lethality, neonatal death and a milder connective tissue disorder (10,11). Mutations of Mnk have been found in some mottled mutants (12,13), but the mutation in the brindled mouse (Mo\textsuperscript{bn}), the closest model of Menkes disease, has not been determined. Northern blots shows that the size and abundance of Mnk mRNA in mutant tissues is the same as normal (12,13). The mutant brindled male is severely copper-deficient at birth, due to defective placental transport of copper (14), and the deficiency is exacerbated by poor intestinal absorption and maldistribution within the body leading to death results ∼17 days after birth (15,16). As with patients with Menkes disease, copper accumulates in some organs, despite the overall copper deficiency. The mutant kidney accumulates 2–3-fold more copper than normal (17) and this accumulation has been attributed to retention of filtered copper in the renal tubular cells (16) due to defective resorption of copper. Excess copper has been located in the proximal tubules of the brindled mouse (18), suggesting that Mnk will be expressed in those tubules; however, the location of this protein has not been reported in any organ. [The gene involved in Menkes disease is known as MNK or ATP7A, the protein product from the human gene is referred to as MNK and the protein product of the mouse Mnk gene is termed Mnk. The corresponding protein products from the Wilson gene (WND or ATP7B) are WND in humans and Wnd in mice.]

The brindled mutant has been used as a model for studies of copper therapy for Menkes disease. Treatment of the mice with copper is very effective provided it is carried out prior to the 10th postnatal day (19,20). The treated mice survive and have a relatively normal life span, even though they continue to manifest signs of copper deficiency such as hypopigmentation. In contrast, copper therapy of Menkes patients has only been successful in a few cases (21) and in many instances has been unsuccessful (reviewed in 22). Kaler has suggested that the type of mutation may influence the
therapeutic response and for good results, some residual Mnk activity may be required (23,24). In this context, the nature of the mutation in the brindled mouse is of particular interest.

In this paper we identify a deletion of two highly conserved amino acids in the ‘stalk’ region of Mnk in the brindled mouse, and use Western blots to show that the mutant kidney contains normal amounts of Mnk, suggesting the mutation does not affect protein stability. We also demonstrate for the first time that Mnk is located in both the proximal and distal tubules of the kidney, and that the distribution is not altered in the mutant kidney. The presence of normal amounts of mutant protein may explain the effectiveness of copper therapy in this mouse model.

RESULTS

Previous work has shown that the levels of Mnk mRNA in tissues from the brindled male mutant were the same as normal, suggesting that the mutation was likely to be a single base change or small deletion (12,13). To identify the mutation in the brindled mouse Mnk mRNA, the coding region from the brindled and normal litter mates was amplified from kidney RNA using RT–PCR, the various PCR products subcloned into plasmid vectors and sequenced as described in the Materials and Methods. Any difference between the mutant and normal sequence was confirmed by repeated RT–PCR and sequencing using RNA isolated from a different mutant animal.

The predicted amino acid sequence of Mnk from the normal litter mate had 14 differences from the published mouse Mnk sequences (12,13), but in every case the amino acid matched one of these sequences, suggesting the differences were either polymorphisms between the various strains of mice used to obtain the previous sequences (Balb/C, DL, C57Bl/6J and ICRXSwiss Webster), or sequencing errors in the previous papers. The sequence from the brindled and normal was identical at each of these positions. The full sequence is submitted to DDBJ/EMBL/GenBank, accession no. U71091. The only difference between the coding sequence of the brindled mouse Mnk and the sequence from the normal litter mate was a deletion of six nucleotides, GCTCTT, from nucleotides 2473 to 2478 (numbering based on our GenBank submission) in the brindled mutant sequence (Fig. 1A). This change results in the deletion of Ala799 and Leu800 with the reading frame being maintained (Fig. 1A). To confirm that this was not a common polymorphism in normal mice, we amplified the region using genomic DNA from various mouse strains; the brindled deletion could be readily identified as an 83 bp PCR product compared with 87 bp from the normal allele (see Materials and Methods). None of the strains examined had the deleted segment: these included C57Bl/6J, CBA, Swiss, BALB/c, DL and mottled blotchy. The same deletion has been found in the brindled Mnk sequence by another group (Barbara Levinson, personal communication). Another similar mottled mutant, the Jax brindled mouse (Mo<sub>brj</sub>) did not have the six base deletion, which is not surprising since it has an independent origin.

The missing amino acids are from the region connecting the 4th transmembrane domain of the protein to the phosphatase or transduction domain in the proposed model of Mnk structure (4). Comparison of this region of a wide range of copper ATPases (Fig. 1B) shows the amino acids to be well conserved from bacteria to man. In particular, the deleted leucine is completely conserved, except for the conservative substitution of isoleucine in the bacterial copper pump, CopA. The conservation of sequence across wide evolutionary distances suggests this region has an important function. None of the disease-causing missense mutations found in MNK or WND, however, are in this region, except for a nonsense mutation at arg795, just out of the 4th transmembrane domain in one patient with Menkes disease (25).

A Western blot of crude membrane proteins extracted from normal and mutant kidney was carried out using an affinity purified antiserum raised against the first 590 amino acids of Mnk (5,26) (Fig. 2, lanes 1 and 2). A band of ~170 kDa, close to the expected size from the predicted amino acid sequence (163 kDa) was detected in both normal and brindled kidneys. This is the first observation of the Menkes protein in tissue
extracts, and confirms the size estimates previously reported in cultured Chinese hamster ovary cells (5). The normal size and quantity of protein in the mutant extracts suggests that the mutation does not affect the stability of the protein. A band of ~95 kDa was seen in the extract from the mutant kidney, this band is occasionally observed in both mutant and normal extracts and may represent a fragment of Mnk formed by proteolysis. The antibody did not detect any proteins in the 170 kDa region in liver (Fig. 2, lanes 3 and 4), a tissue which expresses Wnd but not MNK (6,27) suggesting that the MNK antiserum did not cross-react significantly with Wnd. An unidentified band of ~110 kDa was, however, detected in the liver and may be a related protein but we have not investigated this further. This smaller band was not Wnd since an antibody raised to Wnd detected a band at ~160 kDa in these extracts, but not the 110 kDa band (results not shown).

Since nothing has been reported of the distribution of Mnk in tissues, it was of interest to examine tissue sections from kidney from 10 day old brindled and normal mice using immunohistochemistry (Fig. 3). Staining was observed in both the proximal (P) and distal (D) convoluted tubules, which were distinguished using the PAS stain (Fig. 3C) (28), with the Mnk immunoreactivity showed a marked polarization to the basolateral membrane in the distal tubules. Very little staining was apparent in the glomeruli (G) and preimmune serum gave no staining (not shown). Staining in both normal (A) and mutant (B) kidneys was similar.

**DISCUSSION**

The mutation we describe here in the brindled Mnk is not in any of the recognized functional domains of the protein. Since the only mutation found in either MNK or Wnd in the vicinity of the brindled mutation introduces a premature stop codon (25), there is no information about the possible functional effects of the brindled mutation on the activity of the Cu-ATPase. The Ca-ATPases have been much more extensively investigated, and in these molecules this region forms one of the ‘stalks’, connecting the transmembrane domains with the cytoplasmic loops. The stalks are thought to have an important role in the transmission of conformational changes between the ATP binding site, the aspartic acid which is reversibly phosphorylated and the cation binding sites in the membrane channel and are involved in the E1–E2 transition which is fundamental to the action of P-type ATPases (29). Some mutations in the equivalent stalk of the sarcoplasmic reticulum Ca-ATPase (but not the same amino acids) resulted in molecules which could not undergo the normal conformational changes associated with the E1/E2 transition (30). Mutations of this type characteristically reduce or eliminate Ca transport, but not Ca-dependent phosphorylation of the pump by ATP (29). Based on these studies we propose that the brindled mutation is a conformation change mutant. A mutation in this region of Mnk could be expected to greatly reduce, but perhaps not eliminate Cu transport activity. Some retention of activity is to be expected since complete loss of Mnk results in prenatal lethality in the mouse, as illustrated by the dappled mutant (Mo<sup>d</sup>) (12,13).

The preponderance of staining in the proximal and distal tubules of the kidney is consistent with Mnk having a role in the resorption of copper from urine. The brindled mutant accumulates high copper concentrations in the kidney and the excess copper is located primarily in the proximal tubules (18); since the bulk resorption of many ions is carried out in the proximal tubules, this finding is not surprising. The distribution of Mnk in the proximal tubular epithelial cells appears quite diffuse. Mnk has been localized to the trans-Golgi network of Chinese hamster ovary cells and HeLa cells (26,31), but undergoes striking redistribution in the presence of elevated copper. The results are consistent with Mnk continuously trafficking on vesicles from the trans-Golgi to the plasma membrane, and the intracellular copper concentration influences the rate of outward (trans-Golgi to plasma membrane) or return (plasma membrane to trans-Golgi via endosomes) movement (26). A similar copper-regulated mechanism may be operating in the proximal tubules; however, the resolution of the immunostain is not sufficient to determine if the Mnk is associated with the trans-Golgi or more diffusely distributed.

In contrast to the proximal tubules, the distribution of Mnk in the distal tubules is markedly polarized, with an intense staining pattern associated with the basolateral surface of the cells. The distal tubules in the brindled mouse do not have elevated copper concentrations found in the proximal tubules (31), despite the marked expression of Mnk. The failure of the distal tubules to accumulate copper in the mutant may be explained by the fact that the brindled mouse is suffering from an overall copper deficiency, thus the amount of copper entering the nephron is very low and all of this copper is retained in the proximal tubules gradually accumulating to a high level; the distal tubular epithelial cells, however, remain copper deficient. The marked basolateral accumulation may be a consequence of this copper deficiency, since Mnk in this location would rapidly transport copper back into the circulation. It will be of interest to determine whether the localization of Mnk in the proximal and distal tubules is altered under conditions of copper excess.

The identification of the mutation in the brindled mouse is of importance to our understanding of the function of Mnk and for better understanding of the effectiveness of copper therapy. Cultured cells from the brindled mouse have a very similar efflux defect to that observed with cells from Menkes patients (32) suggesting that the mutant Mnk has very low copper transport activity, despite the normal amounts of protein. The copper transport studies, however, are not accurate enough to distinguish between 0 and 5% residual activity and this difference may be critical for the therapeutic response to copper. As noted above the brindled Mnk presumably retains some activity since complete absence of Mnk results in fetal death in the mouse. Kaler has proposed that successful treatment of Menkes patients with copper will depend on the presence of some residual Cu-ATPase activity, which can transport sufficient copper to allow survival if the block in intestinal transport is by-passed by injection (23,24). Our findings with the brindled mouse are not inconsistent with this hypothesis.

**Figure 3.** Immunohistochemical detection of Mnk in kidney sections. Mnk was detected in paraformaldehyde fixed sections using the MNK antisera and immunoperoxidase staining. (A) Eleven day old normal male; (B) 11 day old mutant male; note the intense staining on the basolateral surface of the distal tubules (D) and the generalized cytoplasmic staining in the proximal tubules (P) in both normal and mutant, the glomerulus shows very little Mnk immunoreactivity; (C) PAS/haematoxylin stain of normal kidney showing the brush border staining in the proximal tubules, the distal tubules are devoid of this brush border staining.
MATERIALS AND METHODS

RT–PCR and sequencing

Total RNA was isolated from the kidneys of normal and mutant 10 day old mice using a modified guanidinium–HCl method (33) and cDNA was synthesized from the RNA using a Boehringer Mannheim kit (ALV reverse transcriptase), by priming with either oligo(dT) or Menkes 2D primers. The cDNA was used as a template in a PCR using mouse Mnk-specific primers as listed below, some with restriction sites added using a Corbett PCR FS96. Each cycle consisted of: denaturation 96°C for 1 min, 1 min annealing at 53°C, 2 min extension at 72°C, for 35 cycles. The PCR products were digested with the appropriate enzyme in some cases using internal restriction enzyme sites, the required bands isolated using agarose gel electrophoresis and subcloned into the appropriate Bluescript II vector. Sequence from the mutant was obtained using an ABI 373 sequencer. Where differences were noted from the published sequences, a second clone from an independent PCR, as well as the normal control was sequenced. In the case of the 6 bp deletion, two clones from two mutants were sequenced as well as two clones from two normal animals.

The primers used for the RT–PCR are shown below. The nucleotides in parentheses are the restriction enzyme sites added to facilitate ligation into the plasmid vectors. The numbers refer to the sequence of the normal mouse Mnk determined in this work, accession no. U71091 except for MMNK-8 numbering which refers to the sequence accession no. U03736. For PCR the primer is paired with the one shown in parentheses.

MMNK-V 54 1 CGCTGCCGCTGCCCCTGC 19 (MMNK-2)
MMNK-1 54 (CGCGA) 242 TTCAGACTCCAAAGACCCTCC 262 (MMNK-2)
MMNK-2 54 (CGCGGATCC) 1642 TTACTTCTGCCTTGCCAGCC 1624 (MMNK-V, MMNK-1)
MMNK-3 54 GCCGAATTCT 1468 TGCCCTCAAGTAATGAGGCC 1487 (MMNK-4)
MMNK-4 54 CGCGAAATAAGCTGATAGGAGCC 2832 (MMNK-3)
MMNK-5 54 CGCGAATTCT 2362 GAGAGAGCCAAAGTGAACCC 2381 (MMNK-6)
MMNK-6 54 3596 CAATGAGAGCCTTTGCGCTG 3579 (MMNK-5)
MMNK-7 54 3258 ACGAAA TAAGA TCCTGGCC 3278 (MMNK-8)
MMNK-8 54 (CGCGGA TCC) 1642 TTACTTCTGCCTTGCCAGCC 1624 (MMNK-V, MMNK-1)

PCR detection of brindled mutation in genomic DNA

Genomic DNA was isolated from livers of normal and mutant mice as previously described (34). Primers were designed on the assumption that the mouse exon containing the mutation would have the same boundaries as the human exon 11 (35). The expected sizes were 87 bp for the normal and 83 bp for mutant. PCR conditions were: 96°C for 5 min, 50°C for 1 min, 72°C for 1 min, for two cycles, then 35 cycles of: 96°C for 1 min, 50°C for 1 min, 72°C for 1 min. Fragments were analyzed on 15% polyacrylamide gels and detected by ethidium bromide staining.

MNK antibody

The antibody was raised against a region of human MNK including the six metal binding sites of MNK (amino acids 1–590) which had been expressed in Escherichia coli. The antibody was affinity purified as described previously (3). MNK and WND have ~46% amino acid identity in this region, so it was considered possible that the antibody raised against MNK may cross react with WND. Since both Mnk and Wnd mRNA are found in mouse kidney (13,36), and the predicted size of WND and MNK are similar (170 and 163 kDa respectively), it was important to confirm the lack of cross-reactivity of the antibody. Using ELISA assay we showed that the antibody gave <2% cross reactivity an expressed region containing four of the metal binding domains of mouse Wnd and having ~50% amino acid identity with the same region of Mnk (results not shown), so we consider Wnd is unlikely to be contributing to the signal on the Western blots and the immunocytochemistry results (see below).

Western blots

About 0.5 g of tissue was homogenized in a ground glass homogenizer at 4°C in 3 ml of 10 mM HEPES pH 7.5, 1 mM EDTA, 250 mM sucrose containing 1 µg/ml aprotinin, 0.5 µg/ml E-64, 0.5 µg/ml leupeptin, 0.4 µg/ml pepstatin, 0.7 µg/ml pepstatin (all protease inhibitors were from Boehringer Mannheim). The homogenate was centrifuged at 100,000 g at 4°C in a Beckman TL-100 centrifuge. The crude membrane pellet was resuspended in 300 µl of 0.06 M Tris–HCl pH 6.8, 10% glycerol, 5% SDS, 5% 2-mercaptoethanol 1 mM EDTA containing the protease inhibitors as above. Proteins were fractionated on a 7.5% SDS–PAGE and the gel was either stained with Coomassie blue or proteins transferred to nitrocellulose using a BioRad Transblot cell. Mnk was detected using the affinity purified antisera at 1:200 dilution (0.3 µg/ml IgG) and the secondary antibody was anti rabbit IgG Peroxidase conjugate (Sigma A-4914) at 1:2000 dilution. Detection was carried out using the Boehringer Mannheim Chemiluminescence procedure (cat. no. 1500 694).

Immunohistochemistry

Kidneys were immersion fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at a thickness of 4µm. Dewaxed sections were blocked with 10% goat serum then incubated with the affinity purified MNK antibody at 1:30 dilution (2 µg/ml) overnight at room temperature. The tissue was incubated with a biotinylated anti rabbit IgG for 1 h at room temperature, followed by avidin/biotin/horseradish peroxidase complex (ABC complex, Vectorstain Elite ABC kit, Vector laboratories), with visualization with diaminobenzidine. Sections were also stained with periodic acid/Schiff’s reagent and haematoxylin.

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REFERENCES


34. Sequence, mapping and disruption of CCC2, a gene that cross-complements the Ca2+ sensitive phenotype of csg1 mutants and encodes a P-type ATPase belonging to the Cys2 ATPase subfamily. Yeast 11, 283–292.