Functional Expression of the Menkes Disease Protein Reveals Common Biochemical Mechanisms Among the Copper-transporting P-type ATPases

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Menkes disease is a fatal neurodegenerative disorder of childhood caused by the absence or dysfunction of a putative P-type ATPase encoded on the X chromosome. To elucidate the function of the Menkes disease protein, a plasmid containing the open reading frame of the human Menkes disease gene was constructed and used to transform a strain of Saccharomyces cerevisiae deficient in CCC2, the yeast Menkes/Wilson disease gene homologue. ccc2A yeast are deficient in copper transport into the secretory pathway, and expression of a wild type human Menkes cDNA complemented this defect, as evidenced by the restoration of copper incorporation into the multicopper oxidase Fet3p. Site-directed mutagenesis demonstrated the essential role of four specific amino acids in this process, including a conserved histidine, which is the site of the most common disease mutation in the homologous Wilson disease protein. The expression of Menkes cDNAs with successive mutations of the conserved cysteine residues in the six aminoterminal MXCXXX metal binding domains confirmed the essential role of these cysteine residues in copper transport but revealed that each of these domains is not functionally equivalent. These data demonstrate that the Menkes disease protein functions to deliver copper into the secretory pathway of the cell and that this process involves biochemical mechanisms common to previously characterized members of this P-type ATPase family.

Copper is a trace element that is essential to human physiology and the development of the central nervous system. This vital role of copper is revealed in patients with Menkes disease, a disorder of copper metabolism characterized by progressive neurodegeneration and death in early childhood (1, 2). Although the pathogenesis of this disorder is poorly understood, the Menkes disease gene has been cloned (3–5) and identified as a member of a unique family of metal-transporting P-type ATPases (6, 7). The Menkes protein is a single-chain 178-kDa polypeptide that is localized to the trans-Golgi network and undergoes a copper-dependent relocation in human and rodent cell lines (8–10). Recent studies have revealed similar findings for the homologous Wilson disease ATPase (11, 12), suggesting a common mechanism for copper transport by these proteins into the secretory pathway of the cell.

Despite these immunocytochemical studies, little data are currently available regarding the function of the Menkes disease protein. Copper-resistant rodent cell lines have been shown to overexpress the Menkes protein, supporting the concept that this protein functions to maintain cellular copper homeostasis (13). Saccharomyces cerevisiae deficient in CCC2, the yeast Menkes/Wilson disease gene homologue (14), are defective in high affinity iron transport due to the lack of copper incorporation into Fet3p, a multicopper oxidase homologous to human ceruloplasmin (15, 16). ccc2A yeast have been utilized to evaluate the function of the Caenorhabditis elegans P-type ATPase (17) as well as to demonstrate copper transport by the Wilson disease protein and to define specific amino acid residues of the protein involved in the delivery of copper into the yeast secretory compartment (11). As the direct connection between holoFet3p biosynthesis and the phenotype of yeast defective in copper transport into the secretory pathway provides an opportunity to assay the function of putative copper-transporting ATPases, the current study employed this approach to define the structure and function relationship of the Menkes disease protein.

EXPERIMENTAL PROCEDURES
Menkes Antibody Production—Oligonucleotide primers synthesized with EcoRI linkers were used to amplify the portion of the Menkes cDNA corresponding to amino acids 1046–1556 in the polymerase chain reaction (PCR) using KlenTaq polymerase (CLONTECH) according to manufacturer’s recommendations. The PCR product was subsequently ligated into pGEX4T-3, confirmed by automated sequencing (Perkin-Elmer), and transformed into Escherichia coli BL21 for protein expression and purification as described (18). Briefly, cultures were grown to an optical density of 600 nm (A600) of 0.6 absorbance units and induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 3 ha t 7 °C.

Hepatocytes were concentrated by centrifugation at 16,000 × g for 20 min at 4 °C. The pellet was redissolved in phosphate-buffered saline (PBS) containing 6 M urea and again centrifuged at 16,000 × g for 20 min at 4 °C. The supernatant was diluted to 1 M urea for 20 min at 4 °C. The pellet was resuspended in phosphate-buffered saline (PBS) containing 6 M urea and again centrifuged at 16,000 × g for 20 min at 4 °C. The supernatant was diluted to 1 M urea with PBS, pooled with the initial soluble fraction, and applied to glutathione-agarose beads. Bound fusion protein was eluted with 5 mM reduced glutathione in 50 mM Tris, pH 8, and dialyzed extensively against PBS, pH 7.4. The purified glutathione S-transferase fusion protein was used to produce rabbit polyclonal antisera (Animal Pharm Services), which was characterized as described previously (8).

Cell Culture, Immunoblotting, and Immunofluorescence—HeLa and HepG2 cells were obtained from the American Type Culture Collection and were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and supplemented with glutamine and penicillin/streptomycin. Cells were lysed in 1% Nonident P-40, 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 10% glycerol supplemented with

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protease inhibitors for 20 min at 4 °C followed by centrifugation for 20 min at 16,000 × g at 4 °C. Proteins (100 µg) from the supernatant were separated by reducing SDS-PAGE and transferred to nitrocellulose by semi-dry transfer (Novablot). Membranes were blocked with 5% milk in PBS and then incubated with a 1:2,000 dilution of antiserum for 1 h at room temperature, followed by several washes with PBS, 0.1% Tween-20. For blocking experiments, antiserum was preincubated with an excess of fusion protein for 2 h at 4 °C. Membranes were then incubated with horseradish peroxidase-coupled secondary antibody, washed, and developed using enhanced chemiluminescence reagent (Amersham Life Science, Corp.) according to the manufacturer’s protocol. For indirect immunofluorescence, cells were grown on coverslips, fixed in ice-cold acetone, and analyzed as described previously (8, 11).

Construction and Mutagenesis of the Menkes cDNA—The coding region of the human Menkes disease gene was obtained by PCR of a human Hela cDNA library (CLONTECH) using oligonucleotides corresponding to known Menkes gene sequence. cDNAs were amplified with KlenTaq polymerase (CLONTECH). The gene was isolated in three portions, and each of the gene fragments was subcloned by T overhang into pCRII (Invitrogen): 1) initiator ATG-BamHI (amino acids 1–591), 2) BamHI-KpnI (amino acids 592–1107), and 3) KpnI-stop codon TAA (amino acids 1108–1500). An XbaI site and consensus Kozak sequence (GCCACC) were engineered into the cDNA immediately preceding the initiator ATG, and an XbaI site was introduced after the stop codon. A silent mutation (T to C) was introduced at nucleotide 6 to abolish the BamHI site at the start of the gene to allow for three-part ligation of the fragment into the XbaI site of yeast vector pVTG6U (19). Site-directed mutagenesis was performed by PCR on the appropriate gene fragment with KlenTaq polymerase and oligonucleotide primer pairs corresponding to the P1001A, H1086Q, A629P, G1019D, and C600 of 0.01 into parallel cultures containing either copper- and iron-deficient media or the appropriate synthetic media. Growth was evaluated after 36–64 h by measuring the A600 of each culture. Growth rates were normalized by dividing the A600 in copper- and iron-deficient media by the A600 in synthetic media and represented as a percentage of wild type growth. The assay for 64Cu incorporation into Fet3p was performed as described (23). Fet3p oxidase activity in membrane extracts was assayed as described (16), and gels were developed from 4 h to overnight in a humid chamber at room temperature and then photographed.

RESULTS

The analysis of the Menkes protein presented in this study was guided by the previously predicted polytopic membrane structure of the protein based on sequence homology and hydropathy analyses (3–5). Fig. 1 presents a schematic topological model of the Menkes protein, which highlights the conserved protein domains. The Menkes protein contains four signature motifs of the P-type ATPases: the phosphatase domain (TGEA), an invariant aspartate residue (DKTGT), a conserved cysteine and proline (CPC) in the proposed cation transduction channel, and an ATP binding sequence (GDGIND). The protein also contains six characteristic copper binding domains at its amino terminus (MXXCXC) as well as a conserved histidine and proline within an SEHPL motif of unknown function, which is present in the homologous Wilson protein and in which a histidine to glutamine alteration is the most commonly identified mutation in patients with Wilson disease (24, 25). In addition, point mutations in the DNA of patients with Menkes disease have been described at positions corresponding to amino acid residues alanine 629 and glycine 1,019 (26).

As the Menkes-specific antiserum previously reported (8) had limited utility in exploring amino-terminal mutations of the Menkes protein, a rabbit polyclonal antiserum was produced against the large cytoplasmic loop of the Menkes protein (indicated by a dotted line in Fig. 1) to detect the expression of the wild type and mutant Menkes proteins within cells. This antiserum recognized a protein of approximately 178 kDa in Hela cell lysates but not in HepG2 cell lysates (Fig. 2), and the protein signal was not observed when the antiserum was preincubated with excess fusion protein (Fig. 2B). A lower nonspecific band at approximately 80 kDa was also detected. Indirect immunofluorescence with the antiserum on Hela cells resulted in perinuclear staining (Fig. 2C), consistent with previous reports on the trans-Golgi localization of the Menkes protein (8–10).

For the structure and function analysis of the Menkes protein, a cDNA encompassing the open reading frame of the...
Menkes gene was constructed as described under “Experimental Procedures.” A yeast expression vector harboring the Menkes cDNA was used to transform a strain of *S. cerevisiae* deficient in CCC2, the yeast Menkes/Wilson disease gene homologue. Immunoblot analysis of lysates fromccc2Δ (IH5) yeast transformed with this Menkes cDNA indicated that the transformed yeast abundantly expressed Menkes protein (Fig. 3A, lane 3). Differential glycosylation or aberrant migration of the protein during SDS-PAGE under the conditions used for the yeast lysate may have accounted for the decrease in apparent molecular weight. To evaluate the function of the expressed protein, wild type (IH4) yeast and ccc2Δ (IH5) yeast transformed with either vector or the Menkes cDNA were pulse-labeled with 64Cu, and copper incorporation into the multicopper oxidase Fet3p was directly detected by autoradiography of membrane extracts separated by nonreducing SDS-PAGE. As demonstrated in Fig. 3B, expression of the Menkes protein in ccc2Δ yeast resulted in copper incorporation into Fet3p (lane 3) similar to the level observed in wild type yeast (lane 1). Since the oxidase activity of Fet3p is copper-dependent, the enzymatic function of Fet3p from the 64Cu-labeled membrane extracts was assayed. Fig. 3C shows that Fet3p oxidase activity paralleled copper incorporation into Fet3p, both in wild type yeast (lane 1) and ccc2Δ yeast transformed with the Menkes cDNA (lane 3). Because the assay of Fet3p oxidase activity accurately reflected the incorporation of copper into the protein, the copper status of Fet3p was evaluated by this method throughout the remainder of the study.

To examine the structure-function relationship of the Menkes protein, point mutations corresponding to the amino acid substitutions P1001A, H1086Q, A629P, and G1019D were introduced into the Menkes cDNA sequence (indicated in boldface in Fig. 1). In addition, the six copper binding domains at the amino terminus of the Menkes protein were mutated from MXCXCC to MXSSXS to create Menkes protein mutants with five, four, three, two, one, or no functional copper binding sites remaining (Fig. 1). Immunoblot analyses of lysates from ccc2Δ yeast transformed with these cDNAs demonstrated that each of the mutant proteins was expressed at a level comparable to the wild type Menkes protein (Fig. 4, lane 2 versus lanes 3–6 and lane 8 versus lanes 9–14).

To evaluate the effect of these structural mutations on Menkes protein function, the growth rate of ccc2Δ yeast transformants in copper-deficient media was examined. Fet3p activity decreases when yeast are grown in copper-deficient media, and this decrease has been correlated to a decrease in growth rate (15). Consistent with these findings, the growth of fet3Δ yeast in copper- and iron-deficient media was 3% that of the growth of wild type (IH4) yeast (Fig. 5A). Since Fet3p activity in ccc2Δ yeast is restored upon expression of functional Menkes protein (Fig. 3C), the growth of ccc2Δ yeast transformed with the mutant Menkes constructs in copper- and iron-deficient media was evaluated. ccc2Δ yeast transformed with vector exhibited 2% of the growth of yeast transformed with the wild type Menkes protein, and the P1001A, H1086Q, A629P, and G1019D mutations all demonstrated reduced growth (5, 11, 47, and 11%, respectively) as compared with yeast expressing the wild type Menkes protein (Fig. 5B). Evaluated through the same assay, the Menkes proteins with mutations in one or two copper binding domains exhibited 83 and 79% that of wild type growth, whereas Menkes proteins with mutations in the first three, four, five, or all six copper binding domains demonstrated negligible growth in the copper- and iron-deficient media (Fig. 5C).

The examination of growth in copper-deficient media suggested that copper-dependent Fet3p activity was compromised in the ccc2Δ yeast expressing the mutant Menkes proteins. To assess copper transport into the secretory pathway by the mutant Menkes proteins more directly, Fet3p oxidase activity in membrane extracts of the transformed ccc2Δ yeast was evaluated. The expression of Menkes mutant proteins P1001A, H1086Q, and G1019D in ccc2Δ yeast did not restore copper incorporation into Fet3p, as evidenced by negligible Fet3p oxidase activity (upper panel of Fig. 6, lanes 5, 6, and 8). In contrast, the A629P substitution in the Menkes protein resulted in a decrease in Fet3p oxidase activity (lane 7) as compared with the wild type Menkes transformant (lane 4), indicating some Fet3p copper incorporation. These results were not due to variations in the amount of Fet3p among transformants, as evidenced by immunoblot analysis of Fet3p on identical samples in B were separated by nonreducing SDS-PAGE and analyzed for Fet3p oxidase activity as described under “Experimental Procedures.”
membrane fractions (Fig. 6, lower panel).

The Fet3p oxidase assay was subsequently used to analyze the role of the copper binding domains in Menkes protein function. Successive rounds of site-directed mutagenesis on the Menkes cDNA produced Menkes mutant proteins defective in one to six copper binding domains as diagrammed in Fig. 7A. Evaluation of Fet3p copper incorporation indicated that expression of the Menkes protein lacking one copper binding domain resulted in Fet3p oxidase activity comparable to that observed with the wild type Menkes protein (upper panel of Fig. 7B, compare lanes 2 and 3), the mutant Menkes protein lacking two copper binding domains resulted in slightly less Fet3p activity (lane 4), and the mutants lacking three to six copper binding domains were devoid of copper-dependent Fet3p oxidase activity (lanes 6–8), despite equal expression of Fet3p between strains as indicated by immunoblot analysis of Fet3p on identical membrane fractions (Fig. 7B, lower panel).

**DISCUSSION**

The data presented in this study demonstrate that the Menkes disease protein functions to deliver copper into the secretory pathway of eukaryotic cells. The ability of the Menkes protein to deliver copper to Fet3p is common to Ccc2p (16) and the Wilson disease protein (11). These findings are consistent with the original observations in Menkes patient fibroblasts of elevated intracellular copper (27, 28), concomitant with reduced activity of the secreted copper-dependent enzyme lysyl oxidase (29). The data are also consistent with recent studies on the localization of the yeast Menkes homologue Ccc2p to a late or post-Golgi compartment (30) as well as immunocytochemical studies that have localized the human and rodent Menkes protein to the trans-Golgi network (8–10) where this ATPase would be positioned to transport copper for incorporation into nascent cupro enzymes within the secretory pathway.

The discovery that the Menkes protein can deliver copper to a ceruloplasmin homologue, a role normally attributed to the Wilson protein in humans and Ccc2p in yeast, indicates that the Menkes protein can interact with upstream and downstream proteins within the Fet3p pathway of copper transport and thus appears to be functionally synonymous with other copper-transporting ATPases. This concept is further sup-
ported by the finding that Menkes proteins harboring the P1001A and H1086Q mutations cannot restore copper incorporation into Fet3p (Fig. 6), comparable to previous findings with the analogous mutants of the Wilson protein (11). The inability of the P1001A mutant to restore Fet3p oxidase activity supports the proposed role of the conserved proline residue in the transduction of copper through the membrane channel formed by the copper-transporting ATPases (7). The H1086Q Menkes mutation parallels the H1070Q mutation in the Wilson protein, the most common point mutation identified in patients with Wilson disease (24). Although the H1086Q mutation is not a known Menkes disease mutation, the similar abrogation of Fet3p activity observed to result from these Wilson and Menkes mutant proteins, together with the conservation of the histidine among all the reported copper-transporting ATPases, suggests that the histidine plays a fundamental structural role in the function of these proteins, perhaps as a site for protein-protein interaction (25). Although the Menkes gene locus demonstrates a propensity toward frameshifts, splice site mutations, and nonsense mutations (26, 31), the data presented here suggest that the spectrum of mutations uncovered by patient DNA analysis may be influenced more by chromosomal dynamics of the gene locus rather than the biology of the transporter, since the most commonly occurring mutation in Wilson disease similarly compromises Menkes protein function. The common function of these ATPases has been questioned by a recent report that certain murine cell types express both the Menkes and the Wilson disease proteins, leading to the proposal of disparate functions for these ATPases (32). The current data, together with recent findings of copper-dependent relocalization for both the Wilson and Menkes proteins in cells, suggest that the physiologically different roles that the Menkes and Wilson proteins assume in human biology may be attributed to the differences in their expression patterns and not differences in cellular function.

The A629P and G1019D disease mutations in the Menkes protein are the only reported missense mutations that occur in predicted soluble domains of the protein (26). Although no function has yet been attributed to glycine 1,019, alanine 629 resides in the “stalk” region of the Menkes protein, which is prone to mutations in Menkes disease and has been proposed to function in joining the copper binding domains to the ATPase core (26). The expression of both the A629P and G1019D mutants in ccc2Δ yeast results in reduced growth in copper-deficient media (Fig. 5) and a decrease but not a deficiency in Fet3p oxidase activity for the A629P mutation (Fig. 6), suggesting that copper transport in patients with this mutation is not absent but only impaired. This finding implies that disease may result when lowered levels of copper transport by the Menkes protein cannot meet the metabolic demands of the cell. The distinction between decreased and deficient copper transport by the Menkes protein mutants may have significant clinical relevance and may in part explain the variable response of patients to copper therapy (33, 34). Alternatively, since in mammalian cells the Menkes and Wilson proteins traffic within the cell in response to the cellular copper concentration (9, 11), these Menkes protein mutants may be defective in copper-dependent trafficking, analogous to observed mutations in the cystic fibrosis transmembrane regulator that affect transport indirectly through mislocalization of the protein (35). Analysis of trafficking by the Menkes protein and its mutants will require expression in mammalian cells, as specific post-Golgi sorting compartments do not appear to be morphologically conserved between yeast and mammalian cells.

Previous studies have demonstrated that a soluble form of the Menkes amino-terminal domain containing the six CXCC motifs binds six molar equivalents of copper, suggesting one copper atom/metal binding motif (36). The current data (Fig. 7) suggest that the Menkes protein may not bind six copper atoms in vivo or that the bound copper atoms are not directly transported through the protein channel. The sudden interruption of copper transport upon mutation of the third copper binding motif, as opposed to a linear decrease in Fet3p activity, suggests either the loss of a critical interaction site or steric constraints in transferring copper from the remaining copper binding motifs to the ATPase core. Steric hindrance is unlikely since Ccc2p and the C. elegans ATPase contain only two and three copper binding motifs, respectively, within a commensurately shorter amino terminus. The potential loss of an interaction site has supporting evidence in recent data demonstrating an interaction between the copper binding motifs of Ccc2p and Atx1p, the copper chaperone to Ccc2p in the yeast Fet3p copper transport pathway (37). A recent study also indicates that mutation of the two cysteine residues in the copper binding domain of the human Atx1p homologue, HAH1 (23), abrogates copper binding by the motif as well as copper delivery to Fet3p (38). The current data may thus suggest that the third copper binding domain plays a critical role in the transfer of copper from HAH1 to the Menkes protein. The finding that the copper binding domains within the Menkes amino terminus are not functionally equivalent despite the conservation of the copper binding amino acids between domains indicates that additional residues must dictate specificity in the transfer of copper between proteins. These residues may reside within or near the copper binding sites or alternatively, may occur distantly within the protein where they would function as a site for protein-protein interaction.

The experiments presented in this study identify the Menkes protein as a member of a mechanistically conserved family of copper-transporting P-type ATPases, which function to transport copper into the secretory compartment of eukaryotic cells. These studies lend insight into pathophysiologic mechanisms in Menkes disease, in which defective placental copper transport during a critical window period in central nervous system development may result in the irreversible neurological deficits observed in Menkes patients. The intracellular transport and export of copper through this family of proteins are likely to occur through an ordered mechanism of copper transfer from the point of cellular entry to exit, guided by specific interactions between proximal proteins within the pathway. Disorders of copper transport caused by mutations within the Menkes protein may thus be classified into categories of functional disruptions affecting chaperone interaction, copper binding, copper transport, or protein trafficking. Further molecular analysis of Menkes protein function based upon this framework may thus provide novel therapeutic approaches to prevent or ameliorate the consequences of disordered copper metabolism in affected patients.

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