Characterization of the Interaction between the Wilson and Menkes Disease Proteins and the Cytoplasmic Copper Chaperone, HAHIp*

(Received for publication, May 24, 1999, and in revised form, July 21, 1999)

Dmitri Larin‡, Constantinos Mekios§, Kamna Das‡, Barbara Ross‡, An-Suei Yang‡, and T. Conrad Gilliam¶¶

From the ¶¶Columbia Genome Center and the ¶¶Departments of Psychiatry and Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Wilson disease (WD) and Menkes disease (MNK) are inherited disorders of copper metabolism. The genes that mutate to give rise to these disorders encode highly homologous copper transporting ATPases. We use yeast and mammalian two-hybrid systems, along with an in vitro assay to demonstrate a specific, copper-dependent interaction between the six metal-binding domains of the WD and MNK ATPases and the cytoplasmic copper chaperone HAHI. We demonstrate that several metal-binding domains interact independently or in combination with HAHIp. HAIH1p, although notably domains five and six of WDp do not. Alteration of either the Met or Thr residue of the HAHIp MTC motif has no observable effect on the copper-dependent interaction, whereas alteration of either of the two Cys residues abolishes the interaction. Mutation of any one of the HAHIp C-terminal Lys residues (Lys56, Lys57, or Lys60) to Gly does not affect the interaction, although deletion of the 15 C-terminal residues abolishes the interaction. We show that apo-HAHIp can bind in vitro to copper-loaded WDp, suggesting reversibility of copper transfer from HAHIp to WD/MNKp. The in vitro HAHIp/WDp interaction is metalospecific; HAHI preincubated with Cu2+ but not with Zn2+, Cd2+, Co2+, Ni2+, Fe3+, or Cr3+ interacted with WDp. Finally, we model the protein-protein interaction and present a theoretical representation of the HAHIp-Cu-WD/MNKp complex.

Copper is an essential trace element that serves as a cofactor for a number of oxygen-processing enzymes involved in diverse biological processes. For example, cytochrome c oxidase is essential for respiration, dopamine β-hydroxylase is essential for catecholamine formation, superoxide dismutase is essential for free radical detoxification, lysyl oxidase is essential for maturation of connective tissue, ceruloplasmin is essential for iron uptake, peptide-α-aminating enzyme is essential for pituitary peptide hormone maturation, and monophenol monoxygenase is essential for melanin synthesis (1–3). Although the precise mechanisms are unknown, copper plays additional roles in hemoglobin synthesis, angiogenesis, nerve myelination, endorphin action, extracellular matrix stabilization, leukocyte differentiation, and neutrophil and granulocyte maturation (3–6). In excess, both cupric and cuprous ions are highly toxic, because they act as electron transfer intermediates and catalyze the formation of hydroxyl radicals. This copper-induced production of reactive oxygen species results in DNA damage, as evidenced by strand breaks and by base oxidation of guanosine within cellular DNA and in lipid peroxidation of membranes, especially in mitochondria and lysosomes (7). Thus, proper copper trafficking is essential to cell vitality.

Dietary copper is absorbed into the body through the intestinal mucosa where it joins recycled endogenous copper secreted into the gastrointestinal tract from other tissues. In general, dietary copper absorption is dependent upon the amount of copper reabsorbed from the fluids of other tissues. Newly absorbed copper is transported to body tissues in two phases. First, albumin, transcuprein, amino acids, and a group of uncharacterized low molecular weight proteins transport the majority of exchangeable copper to the liver. After traversing the basolateral membrane of hepatocytes, copper is distributed to endogenous copper-requiring enzymes and to secreted cuproproteins such as ceruloplasmin, which is thereafter released to plasma for delivery of copper to other tissues. Any excess copper is excreted to the bile by the way of the canalicular (apical) plasma membrane (see Refs. 3 and 4 for reviews).

Two P-type ATPases have recently been characterized in humans, ATP7A and ATP7B (8–12). Mutations in these two genes lead to disorders of copper starvation (Menkes disease) and copper toxicity (Wilson disease), respectively. ATP7A is expressed in all tissues except liver, and mutations in this gene prevent the normal absorption and distribution of copper throughout the body. The resulting copper depletion leads to multi-system disorder and death in childhood. The ATP7B gene is expressed in most tissues but predominantly in the liver. Mutations in this gene lead to excessive copper buildup in the liver. A characteristic feature of WD1 is the abnormally low levels of active (copper-bound) ceruloplasmin. Recent studies show that the molecular components of copper trafficking pathways are highly conserved between yeast and humans. In Saccharomyces cerevisiae, the pathway begins with Cu2+ uptake through the action of copper transporters CTR1p and CTR3p (13, 14). In the cytoplasm, copper is bound by cytoplasmic copper chaperones that then deliver the metal to specific target enzymes (15–21). The ATP1p copper chaperone appears to deliver copper to CCC2p, the yeast homologue of the WD and MNK ATPases (15–17). CCC2p is then required to transfer

1 The abbreviations used are: WD, Wilson disease; MNK, Menkes disease; BCA, bathocuproinedisulfonic acid; PAGE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; Tricine, N-tris(hydroxymethyl)methylglycine; MBP, maltose-binding protein.
Copper trafficking between HAHIp and Wilson's Disease Protein.

Copper from the cytosol to the lumen of the trans-Golgi network where the multi-copper ferrooxidase Fet3p, yeast homologue of ceruloplasmin, is loaded (22, 23).

Lin and Culotta (15) provided the first biochemical evidence for interaction between ATX1p and CCC2p. Based on their subcellular localization, Lin and Culotta (15) suggested that ATX1p works as a cytoplasmic copper carrier protein that delivers copper from CTR1p to CCC2p. Recently, Fuhali et al. (17) used the yeast two-hybrid protocol to demonstrate a copper-dependent interaction between ATX1p and CCC2p.

In this study we show that human homologues of the yeast proteins ATX1p and CCC2p interact in a copper-dependent manner. Using three independent assays we characterize the interaction of HAHIp (Human ATX1-like Homolog) with the homologous Wilson disease (WDP) and Menkes disease (MNKp) proteins. The WD and MNK genes encode six MTCXXC motifs in the N-terminal portion of their proteins, whereas their prokaryotic and yeast counterparts typically encode one or two such motifs (24–26). Using deletion constructs of the WDP metal-binding domain, we provide evidence that a single MTCXXC motif is sufficient to interact with the HAHIp chaperone. Mutagenesis analysis demonstrates that the interaction is dependent on the co-ordination of copper ions by cysteine residues within the MTCXXC motifs of the interacting HAHI and WD proteins. The data suggest a mechanism for the intermolecular transfer of copper ions similar to the "bucket brigade" model previously proposed for the trafficking of toxic ions in bacterial mercury detoxification (27, 28).

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The entire coding region from a human liver HAHI cDNA was amplified by polymerase chain reaction and cloned into the expression vectors pACT2 (CLONTECH), pVP16 (CLONTECH), and pProEx-HTb (Life Technologies, Inc.) to create the plasmid constructs pACT-HAHI, pVP-HAHI, and pHTb-HAHI. To create point mutations within the MTCXXC motif, nucleotide changes were introduced directly into the primers for polymerase chain reaction amplification. Plasmids pACT-WM (without Met), pVP-WM, and pHTb-WM carry HAHI cDNA with the mutation Met10→Val. Plasmids pACT-WC1 (without first Cys), pVP-WC1, and pHTb-WC1 carry an HAHI cDNA with the Cys12→Tyr mutation. Plasmids pACT-WC2 (without second Cys), pVP-WC2, and pHTb-WC2 carry an HAHI cDNA with the Cys15→Tyr mutation. Plasmids pACT-WC2 (without first Cys), pVP-WC2, and pHTb-WC2 carry an HAHI cDNA with the Cys15→Tyr mutation. Plasmids pACT-WC2 (without second Cys), pVP-WC2, and pHTb-WC2 carry an HAHI cDNA with the Cys15→Tyr mutation. Plasmids pACT-WC2 (without first Cys), pVP-WC2, and pHTb-WC2 carry an HAHI cDNA with the Cys15→Tyr mutation. Plasmids pACT-WC2 (without second Cys), pVP-WC2, and pHTb-WC2 carry an HAHI cDNA with the Cys15→Tyr mutation. Plasmids pACT-WC2 (without first Cys), pVP-WC2, and pHTb-WC2 carry an HAHI cDNA with the Cys15→Tyr mutation.

These results indicate that HAHIp interacts directly with the copper ions in bacterial mercury detoxification.

Yeast Two-Hybrid Analysis of the WDp Metal-binding Domain and HAHIp—To test whether HAHIp transfers copper directly to WDP, we used the yeast two-hybrid assay. The entire 68-amino acid coding region of HAHI cDNA was fused in frame to the activation domain of GAL4 (in pACT2 vector), whereas a fragment of WD cDNA encoding 623 N-terminal amino acids was fused in frame to the GAL4 DNA-binding domain (in pS2–1 vector). The WD protein is predicted to possess eight membrane-spanning domains along with four cytoplasmic domains. The six MTCXXC metal-binding motifs are all encoded within the 623-amino acid N-terminal cytoplasmic domain. The two-hybrid plasmids were co-transformed into S. cerevisiae Y187 cells, which contain both the lacZ and his3 reporter genes with upstream GAL4-binding sites. Interaction of the two fusion proteins is necessary to juxtapose the GAL4 DNA-binding and activation domains, which then activate transcription of the reporter genes. Results of these experiments are shown in Fig. 1. The experiment was conducted with vector plasmids only (lane 1), single vector plasmid plus single fusion construct (lanes 2 and 3), both fusion constructs (lane 4), and lamin fusion construct plus HAHIp fusion construct (lane 5). It is clear that the β-galactosidase reporter gene is only activated in yeast cells expressing both the WDp metal-binding domain and HAHIp fusion proteins (Fig. 1, lane 4).

These results indicate that HAHIp interacts directly with the
Copper Trafficking between HAH1p and Wilson’s Disease Protein

**Fig. 1. Yeast two-hybrid assay of the WDp metal-binding domain and HAH1p.** Y187 cells were cotransformed with pAS2–1 and pACT based plasmids, and β-galactosidase activity was measured using o-nitrophenyl-β-D-galactopyranoside as substrate. β-Galactosidase activity is shown as the average of five independent transformants ± S.D. Cells were transformed with the following plasmids (see under “Plasmid Constructs”): lane 1, pAS2–1 (GAL4 DNA-binding domain alone) and pACT2 (GAL4 activation domain alone); lane 2, pAS2–1 and pACT-HAH1 (hybrid of the GAL4 activation domain and HAH1); lane 3, pAS-CBM16 (hybrid of the GAL4 DNA-binding domain and WDp metal-binding domains 1–6) and pACT2; lane 4, pAS-CBM16 and pACT-HAH1; lane 5, pLAM5 (hybrid of the GAL4 DNA-binding domain and the unrelated protein, laminin) and pACT-HAH1; lane 6 is the same as lane 4 except that cells were grown in the presence of 3 mm BCA, a copper chelator.

N-terminal domain of WDp. The strength of signal (2.72 β-galactosidase units) was low relative to positive control plasmids (176.5 β-galactosidase units; data not shown) but much higher than negative controls (0.01–0.04 β-galactosidase units; Fig. 1, lanes 1–3 and 5). We observed the same strength of interaction when the two proteins were cloned in opposite vectors (data not shown). All results obtained from the β-galactosidase assay using o-nitrophenyl-β-D-galactopyranoside as substrate were confirmed both by substituting 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside as substrate and in yeast mating experiments that measure activation of the alternative reporter gene, his3 (data not shown). The presence of 3 μl BCA in the growth medium abolishes the interaction of WDp and HAH1p (Fig. 1, lane 6), suggesting that copper is required for this interaction.

**Characterization of the Six WDp Metal-binding Domains for the Ability to Interact with HAH1p—**In the next set of experiments, we sought to determine which MTCXXC motifs, or combination of motifs, are required for interaction with HAH1p. Results of the yeast two-hybrid assay are shown in Fig. 2. Fusion constructs were generated that contain the full complement of six metal-binding motifs (lane 1) along with various combinations of motifs beginning with the N-terminal most motif 1 to the C-terminal most motif 6: 1–5 (lane 2), 1–4 (lane 3), 5 and 6 (lane 4), 1–3 (lane 5), 2–4 (lane 6), 2 and 3 (lane 7), 1 only (lane 8), 2 only (lane 9), and 3 only (lane 10). The various combinations and numbers of WD metal-binding motifs have only marginal effect on ability to interact with HAH1p with two exceptions. The four N-terminal most motifs (lane 3) promote approximately five times more activity than full length peptide (lane 1), whereas the two C-terminal most motifs generate no evidence of interaction (lane 4). With these two exceptions, various combinations of one, two, or three metal-binding motifs promote virtually the same amounts of β-galactosidase activity. Alteration of the MTCXXC motif abolishes the interaction as shown by comparison of intact motif 1 (lane 9) and motif 1 containing the MTCQSC → ATAQSA alteration (lane 12).

**Characterization of the HAH1p MTCXXC Motif—**In the next set of experiments, we used direct mutagenesis to systematically alter each of the four conserved amino residues in the single MTCXXC motif of the HAH1 protein and then assayed the mutant proteins for copper mediated interaction with WDp. Fig. 3 shows that normal HAH1p (lane 2) and HAH1p with amino acid alterations at MTCXXC (lane 4) and MTCXXC (lane 5) interact with the metal-binding domain of WDp. By contrast, alterations at MTCXXC and MTCXXC residues (lanes 6, 7, 12, and 13) abolish the interaction. We also see in Fig. 3 that omission of the N-terminal (lane 3) or C-terminal (lane 8) 15 amino acid residues of HAH1p likewise disrupts the interaction. In addition to the MTCXXC motif at the N terminus of HAH1p, this protein also harbors three lysine residues at its C terminus, which are highly conserved among eukaryotes. In Fig. 3 we show that mutations Lys86 → Gly, Lys97 → Gly, and Lys106 → Gly do not affect the interaction between HAH1p and WDp as measured by the yeast two-hybrid system (lanes 9–11).

**Assay of HAH1p-Cu-WDp Interaction in Transformed Liver Cells—**To better approximate the in vivo environment of a putative copper trafficking interaction between the HAH1 and WD proteins, we next measured the interaction in HepG2 hepatoma cells. Fragments of the WD cDNA encoding either
Copper Trafficking between HAH1p and Wilson’s Disease Protein

Fig. 3. Yeast two-hybrid assay of the WDp metal-binding domain interaction with HAH1p constructs containing mutations in the MTCXXC motif. Y187 cells were cotransformed with pAS-CBM16 (hybrid of the GAL4 DNA-binding domain and WDp metal-binding domains 1–6) and one of the following plasmids, after which, β-galactosidase activity was measured. Lane 1, pACT2 (GAL4 activation domain alone); lane 2, pACT-HAH1 (hybrid of the GAL4 activation domain and HAH1); lane 3, pACT-HAH-W15N (hybrid of the GAL4 activation domain and HAH1; 15 N-terminal amino acid residues of HAH1 are deleted); lane 4, pACT-HAH-WM (contains the Met16 → Val mutation in HAH1p); lane 5, pACT-HAH-WT (Thr15 → Ala mutation in HAH1p); lane 6, pACT-HAH-WC1 (Cys15 → Tyr); lane 7, pACT-HAH-WC2 (Cys15 → Tyr); lane 8, pACT-HAH-W15C (15 C-terminal amino acid residues of HAH1 are deleted); lane 9, pACT-HAH-K56G (Lys56 → Gly); lane 10, pACT-HAH-K57G (Lys57 → Gly); lane 11, pACT-HAH-K60G (Lys60 → Gly); lane 12, pACT-HAH-C12G (Cys12 → Gly); lane 13, pACT-HAH-C15G (Cys15 → Gly).

623 or 499 N-terminal amino acid residues were cloned into the pM vector to produce fusion products of six and four WDp metal-binding domains, respectively, with the GAL4 DNA-binding domain. The entire coding region of the HAH1 gene was cloned into the pVP16 vector to produce a fusion product consisting of the activation domain of the herpes simplex virus VP16 protein and HAH1p. We co-transfected HepG2 cells with pM and pVP16-based plasmids plus a reporter plasmid (pCAT) containing a CAT gene downstream of four GAL4-binding sites and minimum adenovirus E1b promoter. Interestingly, fusion proteins containing all six metal-binding motifs interact minimally, if at all, with HAH1p (Fig. 4, lane 2), whereas protein encoding the four N-terminal most motifs interact more strongly (lanes 3–5). Interaction between WDp and HAH1p is blocked by altering either cysteine group (lanes 6 and 7) or by deleting either the 15 N-terminal most (lane 8) or 15 C-terminal most (lane 9) amino acid residues of HAH1p. Alteration of the methionine or threonine residue of the MTCXXC motif of HAH1p does not disrupt the interaction (lanes 4 and 5).

In Vitro Assay of the Interaction between the WDp Metal-Binding Domain and HAH1p—Expression constructs were generated that encode first the entire HAH1 coding segment and second a fusion product consisting of the WDp metal-binding domain and the maltose-binding protein (MBP). E. coli SG20050 cells were transformed with these constructs, and the protein products were purified. HAH1p was subsequently incubated with 15 μM CuCl2 in TBS-DTT, followed by removal of unbound copper by dialysis. The MBP-WDp fusion products (Fig. 5A, lanes 4 and 5) or MBP alone (lanes 2 and 3) were bound to amylose resin and incubated with preparations of HAH1p that were either loaded (lanes 3 and 5) or not loaded (lanes 2 and 4) with copper. Bound protein was then eluted with 10 mM maltose and analyzed by SDS-PAGE. Fig. 5A shows that the metal-binding domain of the WD protein binds specifically to HAH1 protein preloaded with copper (lane 5).

In the next experiment (Fig. 5B) the various mutant forms of HAH1p were preincubated with copper and then incubated with amyllose resin-bound MBP-WDp fusion protein. Proteins bound to the resin were subsequently eluted with maltose and analyzed by SDS-PAGE. Consistent with the yeast and mammalian two-hybrid assays results, intact HAH1p (lane 1), MTCXXC modified HAH1p (lane 3), and MTCXXC modified HAH1p (lane 4) were capable of binding the WDp fusion protein, whereas MTCXXC (lane 5) and MTCXXC (lane 6) modified HAH1p and HAH1p missing either the N-terminal (lane 2) or C-terminal amino acids (lane 7) were incapable of binding fusion protein. In this experiment, HAH1p constructs were loaded with copper, whereas resin-bound MBP-Dp fusion protein was copper deficient (see “Experimental Procedures”). The results of the mutagenesis experiments suggest that both Cys residues of the HAH1p MTCXXC motif (copper donor) are required for interaction with WDp (copper acceptor). The inverse experiment with copper-deficient HAH1p mutants and copper loaded resin-bound MBP-WDp fusion generated identical results (Fig. 5C), suggesting that both Cys residues of the HAH1p MTCXXC motif (in this case, copper acceptor) are required for interaction with WDp (copper donor). Thus, four Cys residues, two from the copper donor and two from the copper acceptor, are required for the copper-dependent interaction.

Yeast Two-Hybrid Analysis of the HAH1p/MNKp Interaction—The MNK and WD proteins are highly homologous, sharing 55% amino acid identity (30). Both proteins contain six metal-binding MTCXXC motifs in their N-terminal portion, as well as a signature inter-membrane CPC motif and all characteristic motifs of P-type ATPases. In this set of experiments, we tested whether MNKp interacts with the HAH1p protein using the yeast two-hybrid assay. Fig. 6 (lane 2) shows that the strength of the HAH1p/MNKp interaction is similar to that of the HAH1p/WDp interaction (Fig. 1, lane 4). Addition of 3 mM BCA to the growth medium abolishes the protein-protein interaction (Fig. 6, lane 3), suggesting that copper is required for this interaction. The pattern of MNKp interaction with altered variants of HAH1p (Fig. 6, lanes 4–14) is the same as for the WD protein (Fig. 3). Amino acid alterations at MTCXXC (Fig. 6, lane 5), MTCXXC (lanes 6, Lys56, Lys57, and Lys60 (lanes 10–12) of HAH1p do not affect the HAH1p/MNKp interaction. By contrast, alterations at MTCXXC and MTCXXC (lanes 7, 8, 13, and 14), as well as omission of the N-terminal (lane 4) or C-terminal (lane 9) 15 amino acid residues of HAH1p completely disrupts the interaction.

Metal Specificity of the HAH1p/WDp Interaction—In these experiments purified HAH1p was preincubated with a variety of metal ions. To test metal specificity of the in vitro interaction of the WDp metal-binding domain and HAH1p, we preincubated HAH1p with different metal ions. After removal of unbound metal by dialysis, HAH1p was incubated with amyllose resin-bound MBP-WDp fusion protein. Proteins bound to the resin were eluted with maltose and analyzed by SDS-PAGE. Fig. 7 shows that the metal-binding domain of WDp binds to HAH1 protein that has been preincubated with either Cu2+ or Hg2+ but not with Zn2+, Cd2+, Co2+, Ni2+, Fe3+, or Cr3+.
Copper Trafficking between HAH1p and Wilson’s Disease Protein

**FIG. 4. Measurement of WDp/HAH1p interaction in a mammalian two-hybrid assay.** HepG2 cells were cotransfected with three plasmids, pM-X (fusion of the GAL4 DNA-binding domain and the WDp metal-binding domains; see below), pVP16-Y (fusion of the VP16 protein activation domain and HAH1 mutants; see below), and pG5CAT (harbors the CAT reporter gene). Copper was added to the growth medium to a final concentration of 100 μM. After 4 days the cells were harvested and a CAT enzyme-linked immunosorbent assay was performed using cell lysates. The concentration of CAT detected is proportional to the strength of the interaction between the products of X and Y. CAT concentration is shown as an average for three transfections. Standard deviation does not exceed 10%. Lane 1, untransfected control; lane 2, X = CBM16 (WDp metal-binding domains 1–6), Y = HAH1 (unaltered HAH1); lane 3, X = CBM14 (WDp metal-binding domains 1–4), Y = HAH1; lane 4, X = CBM14, Y = HAH-WM (contains Met50 → Val mutation in HAH1); lane 5, X = CBM14, Y = HAH-WT (Thr51 → Ala mutation in HAH1); lane 6, X = CBM14, Y = HAH-WC1 (Cys12 → Tyr mutation in HAH1); lane 7, Y = CBM14WD, Y = HAH-WC2 (Cys15 → Tyr mutation in HAH1); lane 8, X = CBM14, Y = HAH-W51N (15 N-terminal amino acid residues of the HAH1 are deleted); lane 9, X = CBM14, Y = HAH-W15C (15 C-terminal amino acid residues of the HAH1 are deleted); lane 10 (positive control), cells transfected with pM53 (encodes murine p53) and pVP16-T (encodes SV40 large T-antigen); lane 11 (positive control 2), cells transfected with pM3-VP16 (encodes a fusion of the GAL4 DNA-binding domain and the VP16 protein activation domain).

**FIG. 5. In vitro interaction of the WDp metal-binding domain and HAH1p.** A, HAH1p loaded (lanes 3 and 5) or not loaded (lanes 2 and 4) with copper was incubated with amylose resin-bound fusion product maltose-binding protein-metal-binding domain of WDp (lanes 4 and 5) or amylose resin-bound maltose-binding protein only (lanes 2 and 3) as described under “Experimental Procedures.” Proteins bound to the resin were eluted with 10 mM maltose and analyzed by SDS-PAGE as described under “Experimental Procedures.” Lane 1 shows molecular mass markers. B and C, HAH1p loaded with copper was incubated with amylose resin-bound fusion product maltose-binding protein-WDp metal-binding domain (B) or copper loaded amylose resin-bound fusion product maltose-binding protein-WDp metal-binding domain was incubated with HAH1p (C). Lane 1, unaltered HAH1p; lane 2, HAH1p lacking the 15 N-terminal amino acid residues; lane 3, HAH1p with an alteration Met50 → Val; lane 4, Thr51 → Ala; lane 5, Cys12 → Tyr; lane 6, Cys15 → Tyr; lane 7, HAH1p lacking the 15 C-terminal amino acid residues.

**DISCUSSION**

The Wilson disease gene (ATP7B; WD) encodes a membrane-associated metal transporting P-type ATPase with six metal-binding sites (MTCXXC) located at the N-terminal portion of the protein. The WD gene shares 55% amino acid identity with the Menkes disease gene (ATP7A; MNK), and all major structural and functional motifs are conserved. Both WD and MNK ATPases have been localized to the trans-Golgi network (23, 31–33) where the proteins are presumably engaged in the transfer of cytoplasmic copper to copper-requiring proteins.

This study addresses the mechanism of copper trafficking whereby cytoplasmic copper is delivered to the WD and MNK ATPases. The elaboration of copper trafficking in humans has followed from experiments with highly homologous proteins in the yeast, *S. cerevisiae*. Recent studies in yeast have shown that copper is taken up by cells through the action of CTR1p and then transferred either directly or indirectly to the cytoplasmic chaperone ATX1p. ATX1p is thought to transfer bound copper to CCC2p, which in turn conveys copper to Fet3p. In this study we show that the human homologue of ATX1p, HAH1p, interacts directly with WDp and MNKp using both yeast and mammalian cell assays and in vitro analysis. We further show that two cysteine residues of the HAH1p MTCXXC motif are necessary for the interaction, that a single MTCXXC containing domain of the WD ATPase is capable of interaction with HAH1p, and that copper ion is required for the interaction. We also show that Cu2+, Hg2+, but not Zn2+, Cd2+, Co2+, Ni2+, Fe3+, or Cr3+ can mediate the interaction between the Wilson disease and HAH1 proteins.

Our experiments directly assess the contribution of individual MTCXXC motifs in the interaction between the WD protein and HAH1p. The main conclusion from these studies is that several of the individual WD copper-binding motifs are capable of independent interaction with the HAH1 protein. Metal-binding domains 1, 2, and 3 are each sufficient to interact with HAH1p, and various combinations of domains 1–4 appear to interact with roughly equal efficiency. Interestingly, the last
Copper Trafficking between HAH1p and Wilson's Disease Protein

Two WD metal-binding domains (5 and 6) fail to interact with HAH1p, and the truncated 1–4 domain fragment interacts more strongly than the intact 1–6 domain fragment. The simplest explanation for these results is: first, that the hybrid protein containing WD metal-binding domains 5 and 6 is folded such that the MTCXXC motifs are not exposed on the globule surface and thus are inaccessible for interaction with HAH1p, and second, that under our experimental conditions, the 5–6 fragment masks MTCXXC motifs from the 1–4 fragment. The results indicate that the fifth and sixth copper-binding motifs do not participate directly in the exchange of copper with HAH1p and thus suggest a different function for these motifs.

The interaction of apo-HAH1p with copper-loaded WDP suggests that copper transfer between these proteins is a reversible process. We think that at equilibrium, the HAH1p-Cu-WDP complex can exist with an equal chance of forming either HAH1p-Cu+ + WDP or HAH1p + Cu-WDP. It follows that removal of the Cu-WDP moiety would shift the equilibrium toward the formation of Cu-WDP, whereas removal of HAH1p-Cu would shift the equilibrium toward the transfer of copper from WDP to HAH1p. In vivo, removal of the Cu-WDP complex might be achieved by the transport of copper across the membrane in conjunction with ATP hydrolysis. Thus, our data raise the possibility that HAH1p may function in some circumstances to remove copper from the WD and MNK ATPases, presumably in response to intracellular cues.

In our yeast two-hybrid experiments, HAH1p mutations Lys56 → Gly, Lys57 → Gly, and Lys60 → Gly did not affect interaction between the copper chaperone and either WDP or MNKp. Recent yeast two-hybrid analyses of the yeast HAH1 homologue, ATX1, showed that mutation of the corresponding residues, Lys58 → Glu and Lys61 → Glu + Lys62 → Glu, as well as other compound lysine to glutamate mutations, abolish interaction between the copper chaperon and CCC2p, the WD/MNK homologue (35). As the x-ray crystal structures for ATX1p (36) and the fourth metal-binding domain of MNKp (MNK4) have recently been reported (37), we attempted to reconcile these differences by modeling the WDP/MNKp-HAH1p interaction. As shown in Fig. 8, our model indicates that the HAH1p/MNK4 interaction is stabilized by two salt bridges formed between the positively charged HAH1p lysine residues Lys57 and Lys25, and the negatively charged MNK4 residues Glu22 and Asp63, respectively. According to this model, a Lys60 → Gly mutation would remove one of the stabilizing salt bridges, whereas a Lys57 → Glu mutation would both disrupt the salt bridge and introduce a repulsive force that disrupts the protein-protein interaction. The Lys60 residue is nearly completely exposed to solvent and thus is unlikely to affect protein-protein interaction. The Lys60 residue is close to the copper-binding site as predicted from the ATX1p x-ray structure (36), where it may provide an electrostatic potential gradient that favors the movement of the positively charged copper ion from HAH1p to MNK4. The Lys60 → Glu mutation would reverse the gradient and thus trap the copper at the HAH1p copper-binding motif, whereas a Lys60 → Gly mutation would predictably have less effect.

Proteins containing the MTCXXC motif are present in such evolutionary distant organisms as bacteria and man. The motif consisting of two cysteine residues separated by any two amino acid residues is absolutely conserved among these proteins from diverse phylogenetetic origins. By contrast, the threonine residue is often substituted, and less frequently, MTCXXC containing proteins (for example the CopP protein of Helicobacter pylori) lack a methionine residue at this site. A glycine residue often precedes the MTC motif. Functionally characterized MTCXXC proteins are involved in the transport of either copper or mercury, where the cysteine residues are directly involved in the binding of the metal ions. In all three of our test systems mutation of either Met or Thr did not affect interaction between HAH1p and the metal-binding domain of the WD protein, whereas alteration of either Cys residues abolished the interaction. Interestingly, in the work of Hung et al. (34), mutations in both Cys residues of the MTCXXC motif of ATX1p were required to eliminate copper incorporation into Fet3p. It is possible that the Met residue can provide a second sulfur atom for bi-coordination of copper in ATX1 proteins containing a single Cys in the metal-binding motif.

It is noteworthy that the copper trafficking mechanism first proposed by Lin and Culotta (15) and Pufal et al. (17) from yeast studies and supported by this study of human proteins is closely analogous to the mechanism proposed for mercury detoxification in bacteria (27, 28). In bacteria, the periplasmic protein, MerP, binds Hg2+ with its MTCXXC motif and then relays it to the integral membrane proteins MerT and/or MerC (both contain a pair of cysteine residues believed to be involved in Hg2+ binding) whose function is to convey Hg2+ to the
cytoplasmic mercury reductase, MerA. MerA catalyzes the reduction of Hg\textsuperscript{2+} to the volatile and less toxic Hg\textsuperscript{0} ion. There is an obvious analogy between the MerP → MerT → MerA pathway and the HAH1p → WDp → ceruloplasmin pathway (or ATX1p → CCC2p → Fet3p pathway in S. cerevisiae). Because unbound copper is highly toxic, it is reasonable to propose a bucket brigade like mechanism for copper trafficking.

We do not yet know whether other copper chaperons (39) mediate the transfer of cytoplasmic copper to the MNK and WD ATPases. We do believe that the copper chaperon proteins can, to some extent, exchange copper among themselves. The cytoplasmic copper chaperon Cox 17p is believed to traffic copper to the mitochondria for insertion into cytochrome c oxidase, the terminal oxidase of the respiratory chain (20). Our yeast two-hybrid experiments indicate that HAH1p interacts with Cox17p and that Cox17p, but not HAH1p, interacts with a Cox2p fragment that is presumably located in the mitochondrial intramembrane space (data not shown). Likewise, it is not known how the MNK and WD ATPases transfer copper from the metal-binding domain across the membrane or what role the multiple metal-binding domains play in this regard. The multiple metal-binding domains might serve as traps for copper ions that effectively creates a high local concentration of copper at the intramembrane metal-binding site. Another possibility is that copper binding induces conformational changes in the metal-binding domain that subsequently renders the intramembrane metal-binding site accessible to the cytoplasmic surface. Functional studies will be required to address these questions.

REFERENCES

1. Linder, M. C. (1991) Biochemistry of Copper, Plenum Press, New York

2. Lentie, R. (1984) Copper Proteins and Copper Enzymes, CRC Press, Boca Raton, FL

3. Linder, M. C., Wooten L., Cerveza, P., Cotton, S., Sulzle, R., and Lonelli, N. (1998) Am. J. Clin. Nutr. 67, 965S–971S

4. Turnbull, J. R. (1998) Am. J. Clin. Nutr. 67, 960S–964S

5. Rucker, R., Kosonen, T., Clegg, M. S., Mitchell, A. E., Rucker, B. R., Uruı-Hare, J. Y., and Keen, C. L. (1998) Am. J. Clin. Nutr. 67, 968S–1002S

6. Percival, S. C. (1998) Am. J. Clin. Nutr. 67, 1064S–1068S

7. Bremer, J. (1998) Am. J. Clin. Nutr. 67, 1073S–1110S

8. Tanzi, R. E., Petrukhin, K., Chernov, I., Pellequer, J. L., Wesołowski, S., Ross, B., Romano, D. M., Parano, E., Pavone, L., Brzustowicz, L. M., Devoto, M., Peppercorn, J., Bush, A. I., Sternlieb, I., Pirastu, M., Guccion, J. F., Eryvago, O., Penchaszadeh, G. K., Honig, B., Edelman, I. S., Soares, M. B., Scheinberg, I. H., and Gilliam, T. C. (1993) Nat. Genet. 5, 334–340

9. Petrukhin, K., Fischer, S. G., Pirastu, M., Tanzi, R. E., Chernov, I., Devoto, M., Brzustowicz, L. M., Cayanis, E., Vitale, E., Russo, J. J., Matoceano, D., Boukhgalter, B., Wesołowski, S., Figus, A. L., Loudiaso, J., Cao, A., Sternlieb, I., Eryvago, O., Pirastu, E., Pavone, L., Warburton, D., Ott, J., Penchaszadeh, G. K., Scheinberg, I. H., and Gilliam, T. C. (1993) Nat. Genet. 5, 338–343

10. Bull, P. C., Thomas, G. R., Rommens, J. M., Forbes, J. R., and Cox, D. W. (1993) Nat. Genet. 5, 327–337

11. Yamaguchi, Y., Heiny, M. E., and Gitlin, J. D. (1993) Biochem. Biophys. Res. Commun. 197, 271–277

12. Vulpes, C., Levine, B., Whitney, S., Packman, S., and Gitlicher, J. (1993) Nat. Genet. 3, 7–13

13. Dancis, A., Haile, D., Yuan, D. S., and Klaunzer, R. D. (1994) J. Biol. Chem. 269, 25660–25667

14. Knight, S. A., Labbe, S., Kwon, L. F., Kosman, D. J., and Thiele, D. J. (1996) Genes Dev. 10, 1917–1929

15. Lin, S. J., Pufahl, R. A., Dancis, A., O’Halloran, T. V., and Culotta, V. (1997) J. Biol. Chem. 272, 9215–9220

16. Lin, S. J., Pufahl, R. A., Dancis, A., O’Halloran, T. V., and Culotta, V. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3784–3788

17. Pufahl, R. A., Singer, C. P., Pirasito, K. L., Lin, S., Schmidt, P. J., Fahrni, C. J., Dotson, L. W., Strain, J., Casareno, R. L., Krems, B., and Gitlin, T. C. (1997) J. Biol. Chem. 272, 23469–23472

18. Gierau, D. M., Shtanko, A., and Tzagoloff, A. (1997) Hum. Genet. 99, 329–333

19. Beers, J., Glerum, D. M., and Tzagoloff, A. (1998) Science 285, 1064S–1068S

20. Beers, J., Glerum, D. M., and Tzagoloff, A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 272, 2782–2787

21. Yuan, D. S., Stearman, R., Dancis, A., Dunn, T., Beeler, T., and Klaunzer, R. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2832–2836

22. Yuan, D. S., Dancis, A., and Klaunzer, R. D. (1997) J. Biol. Chem. 272, 25787–25793

23. Odemann, A. S., Custer, H., Krafiz, R., and Solisio, M. (1993) J. Biol. Chem. 268, 12775–12779

24. Francis, M. S., and Thomas, C. J. (1997) Mol. Genet. 253, 484–491

25. Fu, D., Beeler, T. J., and Dunn, T. M. (1995) Yeast 11, 283–292

FIG. 8. Model of the HAH1p-MNK4 complex. This figure shows a side-by-side stereo view of the theoretical HAH1p-MNK4 complex. The MNK4 x-ray crystal has been reported previously (37) and is illustrated in white. Because HAH1p is homologous to MNK4, we adapted the MNK4 structure using the PrISM (protein informatics system for modeling) (38) program to model HAH1p as shown in blue. The complex model was built manually, guided by the common four-helix bundle type folding topology at the interface of the HAH1p and the WD/MNK metal-binding domain and by the distorted tetrahedral complex formed by the copper ion (as shown in magenta) and the four cysteines from the copper-binding motifs of the donor and receptor proteins. Based on this model, HAH1p residue Lys\textsuperscript{77} (K57) is in range to form a salt bridge with the Glu\textsuperscript{22} (E22) residue of MNK4. Glu\textsuperscript{22} is highly conserved in all WD/MNK metal-binding motifs (data not shown), consistent with a critical role in formation of the donor-acceptor complex. The model also predicts the formation of a critical salt bridge between residues Arg\textsuperscript{21} (R21) and Lys\textsuperscript{25} (K25) from HAH1p and Asp\textsuperscript{68} (D63) from MNK4. Negatively charged residues are likewise highly conserved at the Asp\textsuperscript{68} location among WD/MNK metal-binding domains (data not shown). Red depicts negatively charged side groups; blue depicts positively charged groups.
27. Steele, R. A., and Opella, S. J. (1997) Biochemistry 36, 6885–6895
28. Brown, N. L. (1985) Trends Biochem. Sci. 10, 400–403
29. Lutsenko, S., Petrukhin, K., Cooper, M. J., Gilliam, T. C., and Kaplan, J. H. J. (1997) J. Biol. Chem. 272, 18939–18944
30. Petrukhin, K., Lutsenko, S., Chernov, I., Ross, B. M., Kaplan, J. H., and Gilliam, T. C. (1994) Hum. Mol. Genet. 3, 1647–1656
31. Dierick, H. A., Adam, A. N., Escara-Wilke, J. F., and Glover, T. W. (1997) Hum. Mol. Genet. 6, 409–416
32. Nagano, K., Nakamura, K., Urakami, K. I., Umezaya, K., Uchiyama, H., Kiwai, K., Hattori, S., Yamamoto, T., Matsuda, I., and Endo, F. (1998) Hepatology 27, 799–807
33. La Fontaine, S., Firth, S. D., Lockhart, P. J., Brooks, H., Parton, R. G., Camakaris, J., and Mercer, J. F. (1998) Hum. Mol. Genet. 7, 1293–1300
34. Hung, I. H., Casareno, R. L., Labesse, G., Mathews, F. S., and Gitlin, J. D. (1998) J. Biol. Chem. 273, 1749–1754
35. Portnoy, M. E., Rosenzweig, A. C., Rae, T., Huffman, D., O'Halloran, T. V., and Culotta, V. C. (1999) J. Biol. Chem. 274, 15041–15045
36. Rosenzweig, A. C., Huffman, D. L., Hou M. Y., Wernimont, A. K., Pufahl R. A., and O'Halloran T. V. (1999) Structure 7, 605–617
37. Gitschier, J., Moffat, B., Reilly, D., Wood, W. I., and Fairbrother, W. J. (1998) Nat. Struct. Biol. 5, 47–54
38. Yang, A., and Honig, B. (1999) Proteins, in press
39. Valentine, J. S., and Gralla, E. B. (1997) Science 278, 817–818