The Copper Toxicosis Gene Product Murr1 Directly Interacts with the Wilson Disease Protein*

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Ting Y. Tao, Fengli Liu, Leo Klomp, Cisca Wijmenga, and Jonathan D. Gitlin

From the §Edward Mallinckrodt Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri 63110 and the §Department of Medical Genetics, University Medical Center Utrecht, 3583EA Utrecht, Netherlands

Copper toxicosis in Bedlington terriers is an autosomal recessive disorder characterized by excessive hepatic copper accumulation in association with a marked decrease in biliary copper excretion. Recent genetic data have revealed that MURR1, a single copy gene on dog chromosome 10q26, is mutated in this disorder. This gene encodes a 190-amino acid open reading frame of unknown function that is highly conserved in vertebrate species. The Wilson disease protein is a copper transporting ATPase shown to play a critical role in biliary copper excretion. Here we demonstrate that the Wilson disease protein directly interacts with the human homologue of Murr1 in vitro and in vivo and that this interaction is mediated via the copper binding, amino terminus of this ATPase. Importantly, this interaction is specific for this copper transporter, a finding consistent with the observation that impaired copper homeostasis in affected terriers is confined to the liver. Our findings reveal involvement of Murr1 in the defined pathway of hepatic biliary copper excretion, suggest a potential mechanism for Murr1 function in this process, and provide biochemical evidence in support of the proposed role of the MURR1 gene in hepatic copper toxicosis.

Copper is an essential micronutrient that functions as an enzymatic cofactor in diverse metabolic pathways of all aerobic organisms (1). In vertebrates, the liver plays a critical role in copper homeostasis as both the storage site for this metal and the physiologic route for excretion through the biliary system (2). In the liver, hepatocytes are responsible for the uptake and storage of copper, as well as the regulation of excretion of this metal into the bile. The molecular mechanisms determining biliary copper excretion have become clearer following identification of the genetic defect in Wilson disease, an inherited disorder resulting in hepatic copper accumulation. The Wilson disease gene encodes a copper-transporting P-type ATPase localized to the trans-Golgi network of hepatocytes and required for biliary copper excretion (3). With an increase in the hepatocyte cytosolic copper concentration, this protein traffics to a vesicular compartment near the canalicular membrane where copper is accumulated for subsequent excretion into the bile (4–7). The precise mechanisms involved in subsequent copper movement across the canalicular membrane are unknown.

Although Wilson disease is the most common disorder resulting in hepatic copper overload, a distinct form of rapidly progressive cirrhosis associated with a marked increase in hepatic copper content has been described in young infants (8). A similar form of copper-associated cirrhosis is also observed as an autosomal recessive disorder in inbred Bedlington terriers, and studies in these animals reveal a severe impairment in biliary copper excretion (9). Recent molecular genetic analysis in these dogs has revealed an in-frame deletion in MURR1, a gene highly conserved in the vertebrate genome and containing an open reading frame of 190 amino acids with no known protein motifs or homologies (10). We now demonstrate that human Murr1 directly interacts with the Wilson disease protein, providing biochemical evidence in support of the proposed role of the MURR1 gene in hepatic copper toxicosis.

EXPERIMENTAL PROCEDURES

Cloning and Protein Expression—The coding region of Murr1 was amplified by polymerase chain reaction (PCR) from human liver cDNA using oligonucleotide primers corresponding to the 5′ and 3′ sequence of the open reading frame of human Murr1 (9). A hemagglutinin (HA) epitope was included in the 3′-oligonucleotide, and the amplified fragment was ligated into pCDNA3.1 (Invitrogen). An HA epitope was inserted at the carboxyl terminus of human ceruloplasmin (11) and human copper/zinc superoxide dismutase (SOD1) was from David Borchelt. For experiments utilizing glutathione S-transferase (GST) this fragment, absent in the HA epitope, was ligated into pGEX-1 (Amersham Biosciences). pET-32a vector (Novagen) containing amino acids 1–650 corresponding to the copper-binding amino terminus of the human Wilson disease protein as well as recombinant protein produced from this vector were from Amy Rosenzweig. Plasmids used for column binding assays including GST-Atox1, GST-CCS, and pET-32a-CCS and methods for purification of fusion proteins have been described elsewhere (13, 14). Wilson disease protein deletion constructs were amplified by PCR from the full-length cDNA and ligated into pCDNA3.1. The fidelity of all amplified fragments was confirmed by dyeoxy nucleotide sequencing. Pull-down Assays—COS-7 and HepG2 cells were lysed in PBS, 0.5% Triton X-100, 0.5% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride. GST and pET fusion proteins were immobilized on glutathione-agarose beads (Sigma) or His-Bind resin (Novagen) and allowed to interact with COS cell lysate (125 μg of protein). HepG2 cell lysate (100 μg of protein) was used to co-immunoprecipitate human Wilson disease protein as well as recombinant protein produced from this vector were from Amy Rosenzweig. Plasmids used for column binding assays including GST-Atox1, GST-CCS, and pET-32a-CCS and methods for purification of fusion proteins have been described elsewhere (13, 14). Wilson disease protein deletion constructs were amplified by PCR from the full-length cDNA and ligated into pCDNA3.1. The fidelity of all amplified fragments was confirmed by dyeoxy nucleotide sequencing.

1 The abbreviations used are: HA, hemagglutinin; SOD1, copper/zinc superoxide dismutase; CCS, copper chaperone for superoxide dismutase; PBS, phosphate-buffered saline; GST, glutathione S-transferase; DTT, dithiothreitol.

2 To whom correspondence should be addressed: Washington University School of Medicine, McDonnell Pediatric Research Bldg., 660 South Euclid Ave., St. Louis, MI 63110. Tel.: 314-286-2764; Fax: 314-286-2893; E-mail: gitlin@kids.wustl.edu.

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41593
RESULTS AND DISCUSSION

The Wilson disease P-type ATPase transports copper into the secretory pathway of hepatocytes for subsequent excretion of this metal into bile (2, 3). As an inherited deletion in the MURR1 gene results in impaired biliary copper excretion and hepatic copper toxicosis in Bedlington terriers, we sought to determine whether Murr1 interacts with the Wilson disease protein. Pull-down assays were performed utilizing GST-Murr1, GST-CCS, and GST-Atox1 immobilized on glutathione-agarose beads and mixed with lysates from COS-7 cells transiently transfected with a plasmid containing the human Wilson disease cDNA. The inclusion of the copper chaperones CCS and Atox1 served as useful controls as we have previously reported specific interaction of the Wilson disease protein with Atox1 but not CCS using these same methods (13). Following incubation, eluates were subjected to SDS-PAGE and analyzed by immunoblotting with a polyclonal antisera specific for the human Wilson disease protein. As can be seen in Fig. 1A, the Wilson disease protein was readily detected following incubation of GST-Murr1 with this COS cell lysate. The specificity of this interaction was confirmed by detection of the Wilson disease protein following incubation with GST-Atox1 (Fig. 1A, lane 4) and the Wilson disease protein was not dependent upon proteins specific to these cell lysates, as a similar interaction was detected when pull-down assays were repeated using HepG2 cells, a human hepatoblastoma-derived cell line known to express the Wilson disease protein (4). As observed with transfected COS cell lysates (Fig. 1B, lane 4), interaction of HepG2 lysate with GST-Murr1 resulted in readily detectable Wilson disease protein as indicated. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes followed by immunoblotting with Wilson (lanes 1–6) or HA (lanes 1–12) antisera. B–D, immunoprecipitation was carried out with anti-HA antibody using cell lysate prepared from COS cells transfected with Murr1-HA, Wilson protein (WD), or Menkes protein (MNK) as indicated. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes followed by immunoblotting with Menkes (B), HA (C), or Wilson (D) antisera. E, immunoprecipitation was carried out with anti-HA antibody using cell lysate prepared from COS cells transfected with full-length (WD) or carboxyl-terminal deleted (WD(ΔCT)) Wilson protein as indicated. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes followed by immunoblotting with Wilson (lanes 1–3) or HA (lanes 4–6) antisera.

The Wilson disease protein was readily detected following incubation of GST-Murr1 with this COS cell lysate. The specificity of this interaction was confirmed by detection of the Wilson disease protein following incubation with GST-Atox1 (Fig. 1A, lane 4) but not with GST or GST-CCS (Fig. 1A, lanes 1 and 3).

To determine whether a similar interaction occurs in cells endogenously expressing the Wilson disease protein, these pull-down assays were repeated using HepG2 cells, a human hepatoblastoma-derived cell line known to express the Wilson disease protein (4). As observed with transfected COS cell lysates (Fig. 1B, lane 4), interaction of HepG2 lysate with GST-Murr1 resulted in readily detectable Wilson disease protein following analysis of the eluates by immunoblotting (Fig. 1B, lane 6). This interaction between Murr1 and the Wilson disease protein was not dependent upon proteins specific to these cell lysates, as a similar interaction was detected when the pull-down assay was repeated using in vitro translated Wilson disease protein (Fig. 1C, lane 4). The specificity of this interaction was demonstrated by confirming the interaction

(Amersham Biosciences). Equivalent amounts of each protein were then utilized for interaction studies as described previously (13).

Transfection, Coimmunoprecipitation, and Immunoblot Analysis — COS-7, HeLa, and HepG2 cells were obtained from the American Type Culture Collection and grown to confluence in medium with fetal calf serum as described (4, 13). Cells were transfected with pCDNA3.1 containing Murr1, ceruloplasmin, or VDU1 utilizing LipofectAMINE 2000 (Invitrogen) and lysed in 50 mM Hepes, pH 7.5, 0.5% Nonidet P-40, 0.5% Triton X-100, 50 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA supplemented with protease inhibitors, followed by centrifugation for 15 min at 25,000 × g at 4 °C (14). Lysates were precleared overnight with normal rabbit serum and protein A beads (Repligen) followed by immunoprecipitation with affinity-purified rabbit polyclonal antisera to HA (Zymed Laboratories Inc.), human Wilson disease protein, or Menkes disease protein (15). The coimmunoprecipitated complex was released by addition of sample buffer with DTT followed by heating to 100 °C for 5 min, separated by SDS-PAGE, and analyzed by immunoblotting with specific antisera as described previously (4, 13–15). To generate Murr1-specific antisera, a peptide corresponding to amino acids 162–181 of human Murr1 was synthesized, conjugated to keyhole limpet hemocyanin, and injected into rabbits (Covance)

FIG. 1. In vitro interaction of Murr1 and the Wilson protein. A, COS cell lysates expressing the Wilson disease protein were incubated with purified GST-fusion proteins indicated. After washing, bound proteins were eluted, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with antisera to the Wilson protein. B, COS or HepG2 cell lysates were incubated with purified GST fusion proteins indicated. After washing, bound proteins were eluted, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed along with 5% of input (lanes 7 and 8) by immunoblotting with antisera to the Wilson protein. C, in vitro translated 35S-labeled Wilson protein (WD) or SOD1 were incubated with purified GST fusion proteins indicated, and eluates were subjected to SDS-PAGE and analyzed using a PhosphorImager.

FIG. 2. Coimmunoprecipitation of Murr1 and the Wilson protein. A, immunoprecipitation was carried out with anti-HA antibody using cell lysate prepared from COS cells transfected with VDU1-HA, ceruloplasmin (CP-HA), or Murr1-HA either alone or with the Wilson protein (WD) as indicated. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes followed by immunoblotting with Wilson (lanes 1–6) or HA (lanes 1–12) antisera. B–D, immunoprecipitation was carried out with anti-HA antibody using cell lysate prepared from COS cells transfected with Murr1-HA, Wilson protein (WD), or Menkes protein (MNK) as indicated. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes followed by immunoblotting with Menkes (B), HA (C), or Wilson (D) antisera.

E, immunoprecipitation was carried out with anti-HA antibody using cell lysate prepared from COS cells transfected with full-length (WD) or carboxyl-terminal deleted (WD(ΔCT)) Wilson protein as indicated. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes followed by immunoblotting with Wilson (lanes 1–3) or HA (lanes 4–6) antisera.
between Atox1 and the Wilson disease protein (Fig. 1C, lane 2) (13) and CCS and SOD1 (Fig. 1C, lane 7) (14) under these same conditions.

To examine this interaction between Murr1 and the Wilson disease protein in vivo, we performed communoprecipitation of transfected COS-7 cells utilizing a polyvalent rabbit antisera specific for an HA epitope. Following communoprecipitation and washing under gentle conditions, immunoprecipitates were analyzed by SDS-PAGE and the presence of Wilson disease protein confirmed by immunoblotting. Immunoprecipitation of Murr1 from lysates of cotransfected COS cells revealed abundant Wilson disease protein (Fig. 2A, lane 6). This was specific and not the result of the lysis or washing conditions as immunoprecipitation of HA-VDU1 or ceruloplasmin failed to result in detectable Wilson disease protein (Fig. 2A, lanes 3 and 4) even though immunoblotting with antisera to HA (Fig. 2A, lanes 7–12) or the Wilson disease protein (data not shown) reveals equivalent expression of these proteins in the cell lysates.

The specificity of this interaction was next examined by repeating these studies in COS cells transfected with the Menkes disease P-type ATPase. This ATPase is highly homologous in structure and function to the Wilson disease protein (13) and CCS and SOD1 (13, 19) under these same conditions. Although the Menkes disease protein was readily detected in transfected cell lysates (Fig. 2B, lane 6), none was detected following immunoprecipitation of Murr1 (Fig. 2B, lane 3) despite abundant communoprecipitation of the Wilson disease protein (Fig. 2D, lane 2) under these same conditions. In all cases, equivalent expression of Murr1 was observed in each of the transfected cell lysates (Fig. 2C, lanes 1–3). The faint signal detecting Menkes disease protein with the Wilson antisera (Fig. 2D, lane 6) is due to cross-reactivity from overexpression of the Menkes disease protein. The failure to detect interaction between Murr1 and the Menkes disease protein in these experiments supports the concept of a specific functional interaction between Murr1 and the Wilson protein in hepatocytes and is consistent with the liver-specific phenotype of impaired copper homeostasis observed in affected Bedlington terriers (9, 10).

The carboxyl terminus of the Wilson disease protein is predicted to reside in the cytoplasm and contains several motifs that may play a role in the copper-dependent endocytic trafficking of this protein critical to the process of biliary copper excretion (3, 16). As the homologous cytoplasmic tail in the Menkes disease protein has been shown to be required for the trafficking of this protein (17, 18), we next sought to determine whether Murr1 interacts with this region of the Wilson disease protein. Pull-down Wilson disease protein or a construct with a deletion of the entire cytoplasmic tail were co-transfected into COS cells with Murr1 followed by communoprecipitation and immunoblotting as above. As can be seen in Fig. 2E, equivalent interaction was detected between Murr1 and both of these constructs (Fig. 2E, lanes 2 and 3) indicating that the cytoplasmic tail of the Wilson protein is not required for interaction with Murr1. Immunoblotting with antisera to HA (Fig. 2E, lanes 4–6) revealed equivalent expression of Murr1 in these cell lysates.

Previous studies (13, 19–23) have demonstrated that interaction between the cytosolic copper chaperone Atox1 and the amino-terminal region of the Wilson and Menkes disease proteins is essential for copper delivery to the secretory pathway. As immunofluorescence studies indicate that Murr1 is localized to the cytoplasm (10), we sought to determine whether this protein also interacts with Atox1. Pull-down assays were performed utilizing in vitro translated Murr1 and Wilson disease protein. Under these conditions, no interaction was detected between Murr1 and Atox1 (Fig. 3A, lane 3) despite readily detectable Wilson disease protein in eluates from the GST-Atox1 beads (Fig. 3A, lane 4). These in vitro translation reactions again revealed the interaction between Murr1 and the Wilson disease protein observed in previous experiments, and therefore, this method was next utilized to further define the region of the Wilson disease protein specifically interacting with Murr1. As noted previously, full-length in vitro translated Wilson disease protein interacts with Murr1 in this pull-down assay (Fig. 3B, lane 2), and the specificity of this interaction
was confirmed by detection of Wilson disease protein following incubation with GST-Atox1 (Fig. 3B, lanes 4) but not GST or GST-CCS (Fig. 3B, lanes 1 and 3). When this assay was repeated utilizing in vitro translated Wilson disease protein truncated to delete one (WDA1373) or both (WD1010) of the largest cytoplasmic regions containing the ATP-binding domain, the critical aspartate phosphorylation site and the ATPase motif (3, 16), interaction with Murr1 was still detected (Fig. 3B, lanes 7 and 12). As previously, the specificity of this interaction was confirmed by detection of the truncated Wilson disease proteins retaining the amino-terminal domain following incubation with GST-Atox1 (Fig. 3B, lanes 6, 8, 11, and 13).

The carboxyl-terminal deletion studies (Figs. 2E and 3B) suggest that Murr1 may interact with the amino terminus of the Wilson disease protein. To directly examine this possibility, pull-down assays were repeated utilizing a recombinant protein containing all six of the copper-binding motifs in the amino terminus of the human Wilson disease protein. Analysis of the eluates by immunoblotting clearly demonstrated interaction between Murr1 and the Wilson disease protein recovered is largely unaffected by increasing the quantity of GST-Murr1 (data not shown).

To examine this interaction with endogenous Murr1, an antisera to human Murr1 was utilized to detect this protein in cell lysates. As can be seen in Fig. 4B, Murr1 was detected as a single species of 23 kDa present in each of the cell types examined. Analysis of COS cells transfected with HA-tagged Murr1 revealed a second, larger species corresponding to the epitope-tagged transfected protein (Fig. 4B, lane 4). When lysates from HepG2 cells (Fig. 4B, lane 5) were utilized in a pull-down assay with a His fusion protein containing the amino-terminal domain of the human Wilson disease protein, endogenous Murr1 was detected (Fig. 4B, lane 7), confirming the interaction between Murr1 and the Wilson disease protein amino terminus seen above. This interaction was specific as no interaction between Murr1 and the Wilson disease protein was confirmed by detection of Wilson disease protein following incubation with GST-Atox1 (Fig. 3B, lane 4) but not GST or GST-CCS (Fig. 3B, lanes 1 and 3). When this assay was repeated utilizing in vitro translated Wilson disease protein truncated to delete one (WDA1373) or both (WD1010) of the largest cytoplasmic regions containing the ATP-binding domain, the critical aspartate phosphorylation site and the ATPase motif (3, 16), interaction with Murr1 was still detected (Fig. 3B, lanes 7 and 12). As previously, the specificity of this interaction was confirmed by detection of the truncated Wilson disease proteins retaining the amino-terminal domain following incubation with GST-Atox1 (Fig. 3B, lanes 6, 8, 11, and 13).

Inherited loss-of-function mutations in the gene encoding the human Wilson disease ATPase reveal a critical role for this protein in hepatic copper metabolism (24). The Wilson disease protein is localized to the trans-Golgi network of hepatocytes and with increasing intracellular copper concentrations traffics to a cytoplasmic vesicular compartment that distributes near the canalicular membrane (5, 6). While the dynamic processes involved in this localization of the Wilson disease protein as well as the biogenesis and trafficking of these vesicles at the canalicular membrane are not well understood, the data in this current study place Murr1 directly in this known biochemical pathway for biliary copper transport. Recent studies on the molecular mechanisms of the biogenesis and trafficking of lysosome-related organelles have identified ubiquitously expressed, cytosolic proteins with no recognizable homologues in lower eukaryotes, which are essential for the cell-specific formation of melanosomes and platelet dense granules (25). As Murr1 has similar biochemical properties (10), it is reasonable to hypothesize that this protein may function in an analogous fashion to facilitate biliary copper excretion within hepatocytes. In support of such a mechanism, preliminary data indicate that Murr1 may form oligomeric heterocomplexes with other cellular proteins in addition to the Wilson disease protein.2 Taken together, the biochemical findings in this current study indicate a direct role for Murr1 in human biliary copper excretion, consistent with the metabolic observations in Belington terrier copper toxicosis as well as the recent genetic analysis revealing a deletion in the MURR1 gene in affected animals (9, 10).

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