A Single PDZ Domain Protein Interacts with the Menkes Copper ATPase, ATP7A

A NEW PROTEIN IMPLICATED IN COPPER HOMEOSTASIS*

Received for publication, May 31, 2005, and in revised form, July 25, 2005 Published, JBC Papers in Press, July 28, 2005, DOI 10.1074/jbc.M505889200

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The homeostatic regulation of essential elements such as copper requires many proteins whose activities are often mediated and tightly coordinated through protein-protein interactions. This regulation ensures that cells receive enough copper without intracellular concentrations reaching toxic levels. To date, only a small number of proteins implicated in copper homeostasis have been identified, and little is known of the protein-protein interactions required for this process. To identify other proteins important for copper homeostasis, while also elucidating the protein-protein interactions that are integral to the process, we have utilized a known copper protein, the copper ATPase ATP7A, as a bait in a yeast two-hybrid screen of a human cDNA library to search for interacting partners. One of the ATP7A-interacting proteins identified is a novel protein with a single PDZ domain. This protein was recently identified to interact with the plasma membrane calcium ATPase b-splice variants. We propose a change in name for this protein from PISP (plasma membrane calcium ATPase-interacting single-PDZ protein) to AIPP1 (ATPase-interacting PDZ protein) and suggest that it represents the protein that interacts with the class I PDZ binding motif identified at the ATP7A C terminus. The interaction in mammalian cells was confirmed and an additional splice variant of AIPP1 was identified. This study represents an essential step forward in identifying the proteins and elucidating the network of protein-protein interactions involved in maintaining copper homeostasis and validates the use of the yeast two-hybrid approach for this purpose.

Copper homeostasis is essential for the survival and proper functioning of all organisms. The growing number of diseases in which the homeostatic regulation of copper balance is disrupted include Menkes and Wilson diseases as well as neurological diseases such as amyotrophic lateral sclerosis, Alzheimer, Parkinson, and prion diseases (1, 2). Over the last decade, significant progress has been made toward unraveling the biological processes that maintain a fine balance of copper in the body. Several mammalian proteins have been identified and shown to be critical for copper handling. They include metallothioneins, small proteins likely to be involved in the detoxification of excess copper (3); CTR1, a plasma membrane copper uptake protein (4, 5); ATP7A and ATP7B, ATPases required for copper transport to cuproenzymes and excretion of excess copper (6–8); Atox1, a chaperone for copper delivery to the ATPases (9, 10); hCox2, required for copper delivery to Cu/Zn-superoxide dismutase (10); hCox17, Cox11, hSco1, and hSco2, required for copper incorporation into cytochrome c oxidase in the mitochondrial (10, 11); and most recently, MURR1 (COMMD1), a protein implicated in vesicular copper sequestration (12). These proteins probably represent only a small fraction of those that are part of the network of protein interactions that mediate copper homeostasis.

Within the cell, the biological pathways controlling fundamental processes, such as cell growth, cell cycle progression, metabolic pathways, and signal transduction, are initiated and maintained through a series of tightly regulated and coordinated protein-protein interactions (13, 14). Many of these interactions are transient and facilitated by distinct protein interaction domains that recognize specific exposed motifs on partner proteins. For example, Src2 and Src3 homology domains recognize specific motifs that are phosphorylated or proline-rich (14), respectively, whereas PDZ (PSD-95/Discs-large/ZO-1) domains recognize C-terminal motifs (15–17). Among the copper proteins, specific interactions have been demonstrated between each of the ATPases and Atox1 (18, 19) and between ATP7B and MURR1 (20), but none of these interactions appear to involve any of the known interaction modules.

To identify new proteins implicated in copper homeostasis, one approach is to identify interacting partners of proteins known to be essential for copper transport. We have used ATP7A to initiate a search for such interacting proteins. ATP7A is a large transmembrane, copper transporting P-type ATPase that has two roles within a cell: to transport copper to copper-requiring enzymes at the trans-Golgi network (TGN)3 and in the secretory pathway, and at the plasma membrane to efflux excess copper from the cell (7, 8). To fulfill its dual role in the cell, ATP7A maintains a steady state localization at the TGN while constitutively cycling between the TGN and plasma membrane. With elevated intracellular copper, its steady state location shifts to the plasma membrane until copper levels return to normal and the majority of the protein cycles back to the TGN (21–23). Note that in polarized cells, ATP7A traffics to the basolateral surface (24). ATP7A comprises an N-terminal region with six metal binding motifs (GMXGXXC) that each can bind one copper ion, eight transmembrane domains, and several conserved residues or motifs that are characteristic of heavy metal transporting ATPases (19, 25). ATP7A mediates copper translocation across cellular membranes through ATP-driven cycles of phosphorylation.
ion and dephosphorylation, and its copper-induced trafficking is linked to its catalytic activity (7, 8). A diverse array of protein-protein interactions are likely to be integral to the localization and activity of ATP7A.

Ion transport proteins are endowed with multiple sorting motifs. These motifs interact with components of the cell sorting machinery to specify and regulate protein trafficking to and maintenance at specific cell surfaces (e.g. apical versus basolateral), as well as trafficking between intracellular storage compartments and the plasma membrane. Often these motifs are located within cytoplasmic C-terminal tails (26). Whereas many transport proteins employ classical targeting motifs such as tyrosine- and dileucine-based signals, a variety of unique signals are utilized by many proteins for correct targeting (26). Potentially, cells can control the transport activity of ion transporters by regulating the trafficking of these proteins through modulation of protein-protein interactions.

For ATP7A, a C-terminal dileucine motif is required for TGN retrieval (27, 28), whereas a 38-amino acid sequence containing transmembrane domain 3 mediates TGN retention (29). Within the C terminus, there is also a stretch of acidic residues whose role, if any, is unknown, as well as a putative class I PDZ binding motif (1497DTAL1500). Recently, this motif was shown to be required for the targeting and/or retention of ATP7A at the basolateral surface in polarized Madin-Darby canine kidney cells (24). A putative targeting signal also exists within the N-terminal metal binding site 6 (30).

To identify cellular constituents that are likely to play a role in regulating and/or facilitating the copper transport and trafficking activities of ATP7A and, by inference, implicated in copper homeostasis, we have embarked on a study that utilizes the cytoplasmic regions of ATP7A as bait proteins for yeast two-hybrid screening. This approach has been utilized to identify interacting partners of a vast array of proteins including the Na,K-ATPase and gastric H,K-ATPase (31). With the multiplicities of bait proteins for yeast two-hybrid screening. This approach has been used to identify interacting partners of a vast array of proteins includ- ing the Na,K-ATPase and gastric H,K-ATPase (31). With the multiplicities of signals often found within the C termini of proteins, screening of a human brain cDNA library commenced with the ATP7A C terminus as bait. This report focuses on the identification and initial characterization of one of several proteins that were found to interact with the ATP7A C terminus. This protein, designated AIPP1 (ATPase-interact- ing PDZ protein) is a small protein with a single PDZ domain. Co-immunoprecipitation experiments in mammalian cells confirmed the interaction between ATP7A and AIPP1, while mutagenesis experiments showed that AIPP1 interacted within the last 15 amino acids of ATP7A, with no involvement of the dileucine motif. Alternatively spliced transcripts of AIPP1 were identified and potentially encode two isoforms of this protein, one with a putative signal peptide. AIPP1 represents a new protein in copper homeostasis, and its identification provides insights into the mechanism of copper transport and its regulation by cellular constituents.

**EXPERIMENTAL PROCEDURES**

**Bacterial, Yeast, and Mammalian Cell Strains—**Escherichia coli strain DH10B (F-, mcrA, Δ(mrr-hsdRMS-mcrBC), φ80lacZΔM15, ΔlacX74, deoR, recA1, araD, Δ(ara,leu)7697, galU, galK, rpsL, endA1, nupG) was used for cloning, maintenance, and propagation of plasmids. Saccharomyces cerevisiae strain YGH1 (ura3–25 his3–200 ade2–101 lys2–801 trp1–901 leu2–3 C24) was used for cloning, maintenance, and propagation of plasmids. Bacillus subtilis ATCC 6633 and Lactobacillus casei strain (LC12) were used for cloning, maintenance, and propagation of plasmids. Chlorobium limicola strain F13, wild-type strain of C. limicola, was used for cloning, maintenance, and propagation of plasmids.

**Plasmids—**The construction of plasmids has been described previously (35, 36). Plasmid pAS2-1/ATP7A and pAS2-1/ATP7B were kindly provided by Dr. Thomas M. Cavenagh (37). Plasmid pCMB19 containing the ATP7A cDNA (35) as a template. This fragment was ligated into the Ndel/Sall sites of the vector pAS2-1 (Clontech) in frame with the GAL4 DNA binding domain (DNA-BD) to generate plasmid pSLB29.

A fragment encoding the ATP7A C terminus in which the 1487–1488 was converted to 1487–1488 was generated by PCR using pCM300, in which the mutation was previously incorporated (27), as template DNA. PCR using the primers hMNK25 (5′-cccggtgcgcatTTAATATCGAGTGTACATC-3′) and Y2H8 (5′-cccggtgcgcatTTAATATCGAGTGTACATC-3′) introduced a SacI restriction enzyme site (lowercase, boldface type) at nucleotide position 4504 (immediately after the stop codon), and Y2H7, which introduced an Ndel restriction enzyme site upstream of nucleotide position 4210 (amino acid position 1404), led to amplification of a 315-bp product. This PCR product was cloned into the pGEM-TEasy vector (Promega) to generate plasmid pCM354. The insert from this plasmid was isolated with Ndel/PstI and ligated into the corresponding sites of plasmid pAS2-1 to create an in-frame fusion with the GAL4 DNA-BD in plasmid pSLB63.

A bait construct was generated in which the last 15 amino acids were deleted from the ATP7A C terminus (pAS2-1/ATP7A) (1487–1485). PCR amplification was used to generate ATP7A (Δ1485–1450) using the wild-type ATP7A cDNA in pCM319 (35) as a template and oligonucleotides Y2H7 and hMNK23 (5′-cccggtgcgcatTTAATATCGAGTGTACATC-3′) that introduced a stop codon at nucleotide position 4456 (amino acid position 1486) and a PstI site at nucleotide position 4473. The 263-bp PCR product was ligated with the pGEM-TEasy vector and was designated pCM350. The insert was isolated with Ndel/PstI and ligated into the corresponding sites of plasmid pAS2-1 to create a selectable plasmid pSLB65.

For yeast two-hybrid interaction assays with the ATP7B C terminus (ATP7B-C), a 294-bp cDNA fragment that encoded the last 97 amino acids of ATP7B (amino acids 1369–1465) was amplified by PCR using pCM278, containing the wild-type ATP7B cDNA as a template (36), and primers hWNND17 (5′-cccggtgcgcatTTAATATCGAGTGTACATC-3′) and hMNK22 (5′-cccggtgcgcatTTAATATCGAGTGTACATC-3′), which contained Ndel and Sall restriction sites (lowercase, boldface type), respectively. The PCR product was initially cloned into the Ndel/Sall sites of the vector pAS2-1 to generate pSLB13. However, since the ATP7B-C/GAL4 DNA-BD fusion in this vector caused autoactivation of the...
ATP7A-interacting, Single PDZ Domain Protein

reporter genes, the cDNA encoding ATP7B-C was cloned into the pGADGH activation domain vector (Clontech). The insert from pSLB13 was isolated as an EcoRI fragment and ligated into the EcoRI site of pGADGH to create plasmid pSLB20 (pGADGH/ATP7B-C). To carry out interaction assays with ATP7B-C expressed from pGADGH, the AIPP1 cDNA was cloned into pAS2-1. The AIPP1 cDNA was isolated as a BamHI/BglII fragment from the original pACT2-based library plasmid (pSLB56) and ligated into the BamHI site of pAS2-1 in frame with the GAL4 DNA-BD to generate plasmid pSLB75. As an unrelated control for the interaction assays, the plasmid pCMBI52 was used, which encoded the full-length human Atox1 in-frame with the GAL4 activation domain (AD) in pGADGH (19).

Expression constructs to enable the detection of AIPP1 in mammalian cells were generated and encoded AIPP1 with an N-terminal c-Myc epitope tag (EQKLISEEDL). The original plasmid pSLB56 lacked the first 33 bp of the CAT c-myc tag (boldface type and underlined) immediately after the AIPP1 start codon. Primers also incorporated EcoRI/XhoI sites for cloning. PCR products were ligated into the pGEM-T-Easy vector to generate plasmid pSLB81. This plasmid was used as a template with oligonucleotides LOC51248#4F (5'-ccgatctgtgacgccggttccctttatgactacctggcggatatttccttttgctgcccaacaacagccccctggtttttcttgcc-3') and LOC51248#3R to incorporate an N-terminal c-Myc tag (boldface type and underlined) immediately after the AIPP1 start codon. Primers also incorporated EcoRI/XhoI sites (lowercase, boldface type) for cloning. PCR products were ligated into the EcoRI/XhoI site of the mammalian expression vector pcDNA3 (Invitrogen). The final plasmid construct was designated pSLB84.

**Yeas Two-hybrid Screening**—A human brain cDNA library constructed in the GAL4 AD vector pACT2 (Clontech) was screened by co-transformation of the library DNA and the bait construct pSLB29 into *S. cerevisiae* YGHI as previously described (37). Transformants were screened for HIS3 and lacZ gene expression using protocols described in Ref. 70. Positive clones were verified by curing the bait plasmid from the HIS+ lacZ+ positive yeast clones, isolating the library plasmids (s) from Leu+/Trp-/(−lacZ+) auxotrophs, and retransforming library plasmids back into *S. cerevisiae* YGHI either with the pAS2-1 vector alone or with the original bait plasmid pSLB29. Transformants were then reassessed for β-galactosidase activity. For transformants that remained positive for lacZ expression with the bait but negative with the vector alone, plasmid DNA was isolated, transformed into *E. coli* DH10B, and subjected to further detailed analyses.

**DNA Sequence Analysis**—Sequencing of plasmid DNA was carried out either using DYEnamic ET Terminator Chemistry (Amersham Biosciences) with reactions analyzed at the Australian Genome Research Facility (Melbourne, Australia) or using ABI PRISM BigDye Terminator chemistry (Applied Biosystems) with reactions analyzed on an Applied Biosystems 3730S capillary sequencer apparatus at Micromon DNA Sequencing Facility, Monash University (Melbourne, Australia). Sequence analysis and basic sequence manipulation were performed using Sequencher (GeneCodes). Electronic data base searches were conducted using the BLASTN and BLASTP algorithms on the NCBI Web site (www.ncbi.nlm.nih.gov/BLAST/). Domain structure of proteins was investigated using the Simple Modular Architecture Research Tool Web site (smart.embl-heidelberg.de/) or the Conserved Domain Database (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). To determine the signal peptide cleavage sites in amino acid sequences, the SignalP 3.0 Server (available on the World Wide Web at wbs.dtu.dk/services/SignalP/) and PSORT II server (38) were used. Amino acid sequence alignments were carried out using the ClustalX algorithm (39).

β-Galactosidase Filter and Liquid Assays—β-Galactosidase filter and liquid assays on *S. cerevisiae* YGHI transformants were carried out as described in Ref. 70. For the liquid assays, the protocol using chlorophenol red-β-d-galactopyranoside (Roche Applied Science) as the substrate was followed.

**Immunoblot Analysis of *S. cerevisiae* YGHI Transformants**—Total protein extracts from *S. cerevisiae* YGHI transformants were prepared using a trichloroacetic acid extraction method as described in Ref. 70. Approximately 50 μg of protein was subjected to SDS-PAGE (12.5% (v/v) acrylamide). Proteins were transferred to HybBond-C nitrocellulose membrane (Amersham Biosciences) using a Trans-Blot semi-dry electrophoretic transfer apparatus (Bio-Rad) and detected with rabbit-derived antibodies directed against the ATP7A C terminus (diluted 1:500), the GAL4 DNA-BD (2 μg/ml) (Sigma), or the GAL4 AD (1 μg/ml) (Sigma). Secondary antibody used for detection was a goat anti-rabbit IgG peroxidase conjugate (Chemicon). Protein bands were detected after incubation in Lumi-Light Chemiluminescence blotting substrate (Roche Applied Science) and visualized using the Luminescent Image Analyzer LAS-3000 (Raytest Isotopenmessgeraete).

**Mammalian Cell Transfection and Co-immunoprecipitation**—Transient transfection of plasmid DNA into mammalian fibroblast cell lines was carried out using Lipofectamine (Invitrogen) and recommended protocols. Co-immunoprecipitation was carried out essentially as previously described (40). Briefly, cell lysates were prepared from confluent monolayers of transfected or untransfected fibroblasts cultured in a 75-cm² flask as described (40). For immunoprecipitation, 5 μg of either a rabbit polyclonal anti-c-Myc antibody (Sigma) or rabbit preimmune serum was added to an aliquot of each lysate, and ~50 μl of Immunopure immobilized Protein G Plus (50% slurry; Pierce) was added to precipitate immune complexes. Immune complexes were resuspended in Laemmli sample buffer, boiled for 5 min, and fractionated by SDS-PAGE (12.5% and 7.5% (v/v) acrylamide). Proteins were transferred to HybBond-C nitrocellulose membrane (Amersham Biosciences) using a Trans-Blot semi-dry electrophoretic transfer apparatus (Bio-Rad) and detected with either an affinity-purified preparation of a sheep anti-ATP7A antibody (diluted 1:1000) or a polyclonal rabbit anti-c-Myc antibody (Sigma). Secondary antibodies used for detection were a rabbit anti-goat IgG peroxidase conjugate (Chemicon) and a goat anti-rabbit IgG peroxidase conjugate (Chemicon), respectively. Protein bands were detected after incubation in Lumi-Light Chemiluminescence blotting substrate (Roche Applied Science) and visualized using the Luminescent Image Analyzer LAS-3000 (Raytest Isotopenmessgeraete).

**RT-PCR and Northern Blot**—Total RNA was isolated from cell pellets using a combination of TRIZOL reagent (Invitrogen) and RNeasy Mini Kit (Qiagen) following the manufacturer’s recommendations for the RNeasy Mini Kit. For Northern blot analysis, fractionation of ~10 μg of RNA on a 1% (w/v) agarose gel containing 1× MOPS buffer (pH 7) (20 mM MOPS, 2 mM NaOAc, 1 mM EDTA) and 0.66 mM formaldehyde, transfer to a Hybond-N+ membrane (Amersham Biosciences), and hybridization of labeled probe DNA were carried out as previously described (41). Membranes were exposed to an imaging plate (Fujifilm) overnight at 4°C, and bands were visualized using Bio-imaging Analyzer Systems BAS-1800 (Raytest Isotopenmessgeraete). Synthesis of

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*J. F. B. Mercer, unpublished.*
Radiolabeled probes was carried out using [α-32P]dCTP Redivue tip (3000 Ci/mmol) (Amersham Biosciences) and a Random Primed DNA Labeling Kit (Roche Applied Science).

For cDNA synthesis, ~5 μg of total RNA was mixed with 25 ng of random hexanucleotide primers (Roche Applied Science), 0.3 mM dNTPs, 1X avian myeloblastosis virus reverse transcriptase buffer (Roche Applied Science), 2.5 units avian myeloblastosis virus reverse transcriptase (Roche Applied Science) and incubated at 42 °C for 60 min. Approximately 2 μl of this reaction was used for amplification of the cDNA using a MasterTaq Kit (Eppendorf) according to the manufacturer’s recommendations and the appropriate oligonucleotide primers. The reactions were placed in a thermocycler, and the temperature was cycled as follows: 95 °C for 2 min; 95 °C for 20 s, 55 °C for 30 s, 72 °C for 1 min (for a total of 10 cycles); 95 °C for 20 s, 55 °C for 30 s, 72 °C for 2 min (for a total of 20 cycles); 72 °C for 5 min, 5 °C for 10 s.

RESULTS

Identification of an ATP7A-interacting Partner—DNA encoding the entire cytoplasmic C terminus of ATP7A (ATP7A-C), from amino acid 1404 to the stop codon, was cloned into the bait vector pAS2-1 (TRP) to create an in-frame fusion with the GAL4 DNA-BD (pSLB29). To screen for ATP7A-interacting partners, this plasmid was co-transformed into S. cerevisiae YGH1 with a human brain cDNA library constructed in the pACT2 (LEU) vector, which encoded the GAL4 AD. Approximately 3.3 × 10^6 clones were screened on medium lacking histidine, of which 2466 were His+ and 77 were consistently His+/LacZ+. After curing the bait plasmid from the first 20 of these His+/LacZ+ positive clones, analysis of plasmid DNA from the Trp−/Leu+ auxotrophs revealed that two were true positives.

DNA sequence analysis of the inserts from these two plasmids followed by a search of the nucleotide database revealed that they were identical to sequences previously deposited in GenBank™ and encoded uncharacterized proteins. This report focuses on one of these, a hypothetical protein that corresponded to several independent DNA database entries (TABLE ONE) and in this study is encoded by plasmid pSLB56. The ~900-bp insert in this plasmid contained 390 bp of the 423-bp ORF, lacking the first 33 bp, and 486 bp of 3′-untranslated sequence to the poly(A) tail.

In a similar study that utilized the C terminus of the plasma membrane calcium ATPase (PMCA) splice variant 2b as a bait to screen a human brain cDNA library, the same protein was identified as a PMCA-interacting partner and was designated PISP (PMCA-interacting single-PDZ protein) (42). We propose a change in name for this protein to AIPP1 (ATPase-interacting PDZ protein) to reflect its role in interacting with both of these ATPases.

β-Galactosidase filter and liquid assays were carried out on S. cerevisiae YGH1 co-transformants that expressed AIPP1 and wild-type ATP7A-C (pSLB56 + pSLB29), AIPP1 and mutated/truncated ATP7A-C (pSLB56 + pSLB63 or pSLB65), AIPP1 and the ATP7B C terminus (pSLB75 + pSLB20), and AIPP1 and Atx1 as an unrelated protein (pSLB56 + pCMB152). As a control, transformants that contained pAS2-1/ATP7A-C (pSLB29) + pACT2 vector were included. These experiments demonstrated a significant and specific interaction between AIPP1 and ATP7A-C (Fig. 1). The control showed that the positive β-galactosidase result with ATP7A-C and AIPP1 did not arise from nonspecific interaction of ATP7A-C with pACT2 vector-derived proteins. There was no interaction between AIPP1 and the unrelated copper protein Atx1. Significantly, there was no interaction between AIPP1 and the copper ATPase, ATP7B, which is closely related in structure and function to ATP7A. When either the last 52 (not shown) or 15 amino acids were deleted from the ATP7A C terminus, the interaction with AIPP1 was abolished, hence localizing the region of interaction to within the last 15 amino acids of ATP7A (Fig. 1). All of the co-transformants expressed the GAL4 DNA-BD and AD fusion proteins of the expected size, so that the negative β-galactosidase results did not arise through lack of expression of the appropriate proteins (Fig. 1A).

Co-immunoprecipitation experiments were carried out to confirm the interaction between ATP7A and AIPP1 in mammalian cells. An expression construct was generated that encoded the full-length AIPP1 ORF with an N-terminal c-Myc epitope tag. When this construct was transiently transfected into either CHO-K1 cells or human fibroblasts and analyzed for expression of the Myc-tagged protein (AIPP1-Myc) by Western blot analysis with an anti-c-Myc antibody, a protein with an apparent molecular mass of ~17 kDa was detected, consistent with the predicted molecular mass based on its amino acid sequence (data not shown). Therefore, this construct was employed for transfection and co-immunoprecipitation experiments. A clonally pure fibroblast cell line (A12-H9) was originally derived from a Menkes patient and was previously immortalized and transfected to stably express the ATP7A protein (34). For co-immunoprecipitation experiments, this cell line was transfected to transiently express AIPP1, after which a total cell lysate was prepared and incubated with either a rabbit anti-c-Myc antibody or rabbit preimmune serum as a control. SDS-PAGE analysis of the immune complexes precipitated with Protein G beads showed that the anti-c-Myc antibody precipitated both AIPP1-Myc and ATP7A

### Table ONE

| GenBank™ accession number | Total length | 5′-UTR | 3′-UTR | Protein accession number | Protein length | Source/Reference |
|--------------------------|-------------|-------|-------|--------------------------|---------------|-----------------|
| NM_016484                | 1712        | 176   | 1115  | NP_057568                | 140           | Ref. 68         |
| AF151061                 | 980         | 72    | 486   | AAP36147                 | 140           | CD34+ stem cell, Ref. 69 |
| AK024746                 | 977         | 70    | 486   | NA                       | NA            | Primary smooth muscle cells of human coronary artery |
| BX537725                 | 1712        | 176   | 1115  | CAD97820                 | 140           | Human cerebellum |
| BC012996                 | 948         | 41    | 486   | AAH12996                 | 140           | Rhabdomyosarcoma (muscle) |
| CR457149                 | 423         |       |       | CAG33430                 | 140           | Hypothetical protein LOC51248 |
| AY358829                 | 1206        | 0     | 486   | AAQ89188                 | 172           | Ref. 68         |
| BC089433                 | 906         | 0     | 486   | AAH89433                 | 139           | Chondrosarcoma lung metastasis |
from the lysate, whereas the preimmune serum did not precipitate either protein (Fig. 2). We concluded that the interaction between ATP7A and AIPP1 represented a true interaction in a mammalian cell environment.

The ATP7A-C-AIPP1 Interaction Is Not Dependent on Copper Levels in S. cerevisiae—Within AIPP1 and the C terminus of ATP7A, there were no identifiable metal binding motifs or ligands likely to bind copper, so we did not expect the interaction to be copper-dependent. Surprisingly, when yeast transformants that expressed ATP7A-C and AIPP1 were cultured under conditions of copper depletion, by the addition of BCS and copper-treated cultures showed an unexpectedly large increase in the extent of the interaction (Fig. 3). The addition of 50 μM copper (as CuSO4) to copper-depleted cells decreased the interaction to basal levels (Fig. 3). Higher concentrations of copper to 500 μM were tested and yielded similar results (data not shown). However, Western analysis of BCS and copper-treated cultures showed that there was a detectable increase in the levels of expression of the GAL4 DNA-BD/ATP7A-C and GAL4 AD/AIPP1 fusion proteins as copper was depleted from the medium and a significant decrease when copper was added back (Fig. 3). This observation suggested that the increase in β-galactosidase activity with copper depletion could be attributed to increased protein expression levels and that the interaction between ATP7A-C and AIPP1 was not copper-dependent. A possible explanation for the increased protein levels is that copper may compete with zinc for binding to transcription factors, so that depletion of cellular copper enhances transcription factor activity, leading to increased protein expression levels and consequently increased β-galactosidase activity. Our observations that the addition of zinc to BCS-treated cultures caused consistently high levels of β-galactosidase activity (at least 2-fold that observed in BCS-treated cultures) (data not shown) is consistent with this possibility.

AIPP1 Is a Single PDZ Domain Protein—Protein sequence analysis of AIPP1 failed to identify any predicted transmembrane helices, but identified a putative PDZ domain (Fig. 4). The PDZ domain comprises 96 amino acids of the 140 amino acids of AIPP1 and was the region that showed greatest similarity in data base searches with other PDZ domain-containing proteins. Other single PDZ domain proteins with a high level of similarity to AIPP1 included the MALS/VELIS family of proteins, which are the mammalian homologues of Caenorhabditis elegans Lin7, the Golgi-associated PDZ and coiled-coil motif-containing protein (GOPC), and...
common to both transcripts. Two transcripts were identified, a minor one at ~1.4 kb and a major form at ~1.1 kb (Fig. 5A). Based on the length of the cDNA sequences deposited in GenBank™, the AY358829 cDNA may be derived from the minor transcript, whereas AF151061, AK024746, and BC012996 are likely to be derived from the major transcript. The cDNAs corresponding to NM_016484 and BX537725, although encoding the shorter AIPP1 isoform, are significantly longer (>1.7 kb) due to an extended 3' untranslated region arising from an alternative polyadenylation start site. A transcript corresponding to these cDNAs was not detected, either due to a much lower abundance or due to tissue-specific expression. In the same experiment, the effect of intracellular copper levels on AIPP1 transcript abundance was investigated. Total RNA was isolated from a Menkes patient fibroblast line (Me32a-T22/2L), and the same cell line transfected to stably express ATP7A (A12-H9), which were previously shown to have high and low intracellular copper levels, respectively (34, 43). Hybridization of these RNAs with the AIPP1 cDNA probe fragment revealed that AIPP1 transcript levels were not affected by variation in intracellular copper concentrations (Fig. 5A).

To further verify the existence of the alternatively spliced products in vivo, RT-PCR was carried out on total RNA using oligonucleotide primers complementary to common regions of the AIPP1 cDNA sequences (Fig. 5B). A shorter major product and two less abundant products were amplified. Sequence analysis revealed that the shorter, most abundant product (602 bp) represented AIPP1a, whereas the largest and least abundant product (841 bp) represented AIPP1b. A third product (674 bp) was also amplified and encoded AIPP1a but contained additional 5' untranslated sequence. This splice product was designated AIPP1a'. The existence of AIPP1a and AIPP1b were confirmed by RT-PCR using several primer combinations that included those specific for AIPP1b (data not shown). Fig. 5C shows a schematic representation of the intron-exon arrangement from which AIPP1a and AIPP1b are derived.

**DISCUSSION**

The molecular dissection of ATP7A to identify amino acid residues and signals important for its copper translocation and copper-induced trafficking activities is an ongoing process that has, over the last decade, yielded significant insight into the mechanism of action of this transport protein. N- and C-terminal as well as transmembrane sequences have been implicated in regulating its subcellular localization (27–30, 44), whereas the N-terminal metal binding motifs as well as those residues that are highly conserved and characteristic among the ATPases are required for its copper transport activity (45–48). Protein kinase-dependent phosphorylation also may be implicated in regulating the localization and/or activity of ATP7A (49). However, the proteins in the cell that act upon and interpret these various signals to regulate and/or facilitate the activities of ATP7A have not been identified.

This study represents part of a larger project whose objective is 2-fold: 1) to identify proteins that interact with ATP7A to provide insight into the mechanisms and interactions that regulate its activities and 2) through the identification of ATP7A-interacting partners, to identify other proteins important in the maintenance of copper homeostasis. With a number of C-terminal signals already identified within ATP7A, the search for interacting partners commenced with a yeast two-hybrid approach in which the entire C terminus was used as a bait to screen a human brain cDNA library.

We identified a new protein, AIPP1, implicated in copper homeostasis through its interaction with ATP7A. AIPP1 is a small, 140-amino acid protein composed mainly of a single PDZ domain. PDZ domains were first recognized in three proteins (the postsynaptic density protein, PSD-95, the Drosophila septate junction protein, Discs-large, and the
Amino acid sequence alignment of AIPP1 and other single PDZ domain proteins.

Human proteins with similarity to AIPP1 were aligned using the ClustalX algorithm (39) and BioEdit software (67). Only the AIPP1b sequence was used in the alignment, since it is identical to AIPP1a except for the additional 32 N-terminal amino acids. The predicted signal peptide cleavage site for AIPP1b is indicated by an arrow. Residues that are identical or similar in the majority (60%) of sequences are shaded in black and gray, respectively. The PDZ domain is boxed, and the conserved "GLGF" motif is indicated by asterisks. GenBank™ protein accession numbers are as follows: AIPP1b, AAQ89188; GOPC, NP_065132; H92522-Syn (basic syntrophin isoform b), NP_570896; MALS-1, NP_004655; MALS-2, AAQ89104; MALS-3, NP_060832. The numbers on the left indicate the amino acid position within the sequence.

Figure 3. The effect of copper on the interaction between AIPP1 and the ATP7A C terminus. S. cerevisiae YGH1 co-transformants expressing AIPP1 and the wild-type ATP7A C terminus (ATP7A-C) were passaged for two nights in normal growth medium, in medium supplemented with the copper chelator BCS (Sigma), or in medium supplemented with BCS and CuSO4 at the concentrations indicated. β-Galactosidase activity was measured using chlorophenol red-β-galactopyranoside as the substrate. Within a single experiment, the β-galactosidase activity of triplicate samples was measured. The values shown represent β-galactosidase activity calculated in Miller units and using the average value obtained from at least three independent experiments ± S.D. The data were analyzed by Student’s t test, and significant differences (p < 0.05) were found for the following pairs: *, 0 μM BCS plus 0 μM copper versus 100 μM BCS plus 0 μM copper; ***, 100 μM BCS plus 0 μM copper versus 100 μM BCS plus 50 μM copper. The panels below the graph show Western blots of the GAL4 DNA- BD/ATP7A-C and GAL4 AD/AIPP1 fusion proteins that are expressed in each culture represented in the graph. Protein extracts from the untreated and treated cultures were subjected to SDS-PAGE and Western blotting with an ammonium sulfate-precipitated preparation of antibodies directed against a peptide within the ATP7A C terminus (diluted 1:500) or with affinity-purified GAL4 AD antibodies (1 μg/ml) (Sigma). An image of the Ponceau Red-stained membrane is also shown to demonstrate approximately equivalent protein loading in each lane.

Figure 4. Amino acid sequence alignment of AIPP1 and other single PDZ domain proteins. Human proteins with similarity to AIPP1 were aligned using the ClustalX algorithm (39) and BioEdit software (67). Only the AIPP1b sequence was used in the alignment, since it is identical to AIPP1a except for the additional 32 N-terminal amino acids. The predicted signal peptide cleavage site for AIPP1b is indicated by an arrow. Residues that are identical or similar in the majority (60%) of sequences are shaded in black and gray, respectively. The PDZ domain is boxed, and the conserved "GLGF" motif is indicated by asterisks. GenBank™ protein accession numbers are as follows: AIPP1b, AAQ89188; GOPC, NP_065132; β2-Syn (basic syntrophin isoform b), NP_570896; MALS-1, NP_004655; MALS-2, AAQ89104; MALS-3, NP_060832. The numbers on the left indicate the amino acid position within the sequence.
epithelial tight junction protein, ZO-1) and are among the most common protein interaction domains in organisms (15, 17). The vast majority of proteins that contain PDZ domains are associated with the plasma membrane (50). PDZ proteins have an essential role in transporting and maintaining the formation of functional protein complexes in these complexes to distinct subcellular locations, as well as organizing and targeting transmembrane, membrane-associated, and cytosolic protein complexes to the apical surface and intracellularly under conditions of elevated copper. When basal copper conditions were restored, ATP7A cycled back to the Golgi area (24). These data suggested that the PDZ binding motif from this study and that of Greenough et al. (24) suggest that AIPP1 is likely to represent the PDZ protein that interacts with DTAL of ATP7A at the basolateral membrane under elevated copper conditions. Taken together, data from this study and that of Greenough et al. (24) suggest that AIPP1 is likely to represent the PDZ protein that interacts with DTAL of ATP7A to target and/or stabilize ATP7A at the basolateral membrane under elevated copper conditions.

Recently, the C terminus of PMCA2b was used as a bait to screen a human brain cDNA library for interacting proteins (42). A protein iden-
If AIPP1 functions similarly to MALs and other PDZ proteins, then it is likely to interact with other proteins in the cell in mediating the transport, targeting, or retention of its interacting partners at the basolateral surface. The C terminus of the closely related copper ATPase ATP7B did not interact with AIPP1, and a putative PDZ binding motif, according to current classification systems, could not be identified at its C terminus. This was not an unexpected result, since ATP7B localizes differently from ATP7A, toward the apical membrane of hepatocytes under elevated copper conditions (64–66).

Disruption of PDZ domain interactions is known to result in disease phenotypes (17). For example, a deletion mutation that removes the last three amino acids of CFTR accounts for the symptoms of cystic fibrosis (CF) in a minority of CF patients (26). It is feasible that disruption of the ATP7A/AIPP1 interaction could cause a Menkes-like, copper deficiency phenotype. In this context, it is interesting to note that the AIPP1 gene is located on the X chromosome (Xq13.1), in the vicinity of the ATP7A locus at Xq13.2-Xq13.3, so that mutations in this gene, as with Menkes disease, would affect predominantly males. Hence, AIPP1 offers itself as an additional candidate in cases where patients might present with such symptoms but in whom a mutation in ATP7A cannot be identified.

The identification of the novel PDZ protein, AIPP1, as an interacting partner of ATP7A represents an important first step toward discovering new proteins in copper homeostasis and offers an exciting development in the study of ATP7A trafficking in the cell. The added benefit of discovering new proteins that modulate copper homeostasis is that these molecules represent potential new targets for therapy in disorders where copper balance is disrupted, and they could potentially serve as candidates for study in disorders of copper transport for which the genetic basis has not yet been uncovered.

Acknowledgments—We thank Michael Petris for supplying the plasmid construct containing the ATP7A LL-AA mutation, Yolanda Deal for invaluable technical support, and Michael Cater for helpful scientific discussions and critical reading of the manuscript.

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A Single PDZ Domain Protein Interacts with the Menkes Copper ATPase, ATP7A: A NEW PROTEIN IMPLICATED IN COPPER HOMEOSTASIS
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J. Biol. Chem. 2005, 280:33270-33279. doi: 10.1074/jbc.M505889200 originally published online July 28, 2005

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