Cellular copper levels determine the phenotype of the Arg⁸⁷⁵ variant of ATP7B/Wilson disease protein

Arnab Gupta^{a,1}, Ashima Bhattacharjee^{a,1}, Oleg Y. Dmitriev^b, Sergiy Nokhrin^b, Lelita Braiterman^c, Ann L. Hubbard^c, and Svetlana Lutsenko^{a,2}

Departments of ^aPhysiology and ^cCell Biology, The Johns Hopkins University, Baltimore, MD 21205; and ^bDepartment of Biochemistry, University of Saskatchewan, Saskatoon, SK, Canada S7N 5E5

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In human disorders, the genotype-phenotype relationships are often complex and influenced by genetic and/or environmental factors. Wilson disease (WD) is a monogenic disorder caused by mutations in the copper-transporting P-type ATPase ATP7B. WD shows significant phenotypic diversity even in patients carrying identical mutations; the basis for such diverse manifestations is unknown. We demonstrate that the 2623A/G polymorphism (producing the Gly⁸⁷⁵→Arg substitution in the A-domain of ATP7B) drastically alters the intracellular properties of ATP7B, whereas copper reverses the effects. Under basal conditions, the common Gly⁸⁷⁵ variant of ATP7B is targeted to the *trans*-Golgi network (TGN) and transports copper into the TGN lumen. In contrast, the Arg⁸⁷⁵ variant is located in the endoplasmic reticulum (ER) and does not deliver copper to the TGN. Elevated copper corrects the ATP7B-Arg⁸⁷⁵ phenotype. Addition of only 0.5–5 μ M copper triggers the exit of ATP7B-Arg⁸⁷⁵ from the ER and restores copper delivery to the TGN. Analysis of the recombinant A-domains by NMR suggests that the ER retention of ATP7B-Arg⁸⁷⁵ is attributable to increased unfolding of the Arg⁸⁷⁵-containing A-domain. Copper is not required for the folding of ATP7B-Arg⁸⁷⁵ during biosynthesis, but it stabilizes protein and stimulates its activity. A chemotherapeutic drug, cisplatin, that mimics a copper-bound state of ATP7B also corrects the “disease-like” phenotype of ATP7B-Arg⁸⁷⁵ and promotes its TGN targeting and transport function. We conclude that in populations harboring the Arg⁸⁷⁵ polymorphism, the levels of bioavailable copper may play a vital role in the manifestations of WD.

trafficking | cisplatin | phenotypic variability

Wilson disease (WD) is an autosomal recessive disorder, caused by mutations in the copper-transporting ATPase ATP7B. WD manifests as hepatolenticular degeneration associated with copper accumulation in the liver and other organs (1). Although WD follows a simple Mendelian pattern of inheritance (monogenic disorder), the phenotypic manifestations of WD are diverse and strong genotype-phenotype correlations have not yet been found. Different severity of the disease and a variable time of onset for the same causative mutations suggest involvement of additional genetic and/or environmental factors (2). Furthermore, several ATP7B mutations have a dual designation as a “disease variant” and “nondisease variant” depending on the population in which they were identified. One such substitution is Gly⁸⁷⁵→Arg (c2623A/G) within the A-domain of ATP7B (Fig. S1A).

Arg at position 875 was originally detected in the ATP7B sequence generated using the cDNA library from the brain of a patient with a neurological (non-WD) disease (3). Parallel studies using a human kidney cDNA library identified Gly at this position (4); Gly is also conserved in ATP7B orthologs (Fig. S1B). In vitro, both the Arg- and Gly-ATP7B variants hydrolyze ATP with the formation of a phosphorylated intermediate (5, 6). This observation is consistent with the designation of c2623A/G as a polymorphism, which was made in genetic studies of the Han Chinese population (7). In the Indian population, however, Arg⁸⁷⁵ is a WD-causing mutation (8). To identify reasons for this dual behavior, we investigated the intracellular targeting and activity of ATP7B-Arg⁸⁷⁵ under various conditions. We demonstrate that

unlike the common Gly variant, ATP7B-Arg⁸⁷⁵ is exquisitely sensitive to variations in available copper levels, which modulate its properties. We also found that a well-known chemotherapeutic drug, cisplatin (DDP), can mimic effects of copper and improve the “disease-like” phenotype.

Results

ATP7B-Arg⁸⁷⁵ Does Not Show Transport Activity Under Basal Conditions. The physiological function of human copper-ATPases is to transport copper into the *trans*-Golgi network (TGN) for biosynthetic incorporation into copper-dependent enzymes, such as ceruloplasmin, tyrosinase, or dopamine- β -hydroxylase. To evaluate the transport activity of ATP7B-Arg⁸⁷⁵, we used Menkes fibroblasts transfected with tyrosinase (YSTT cells). These cells lack endogenous copper-ATPase and are unable to transport copper into the TGN, thus producing inactive tyrosinase (9, 10). The expression of ATP7B-Gly⁸⁷⁵ in YSTT cells restores copper delivery to the TGN and activates tyrosinase, as evident from the formation of black pigment [levo-3,4-dihydroxy-L-phenylalanine (L-DOPA) quinone]. In contrast, ATP7B-Arg⁸⁷⁵ does not activate tyrosinase (Fig. 1A) even though ATP7B-Arg⁸⁷⁵ is expressed in cells (Fig. 1B). Thus, under standard conditions, ATP7B-Arg⁸⁷⁵ does not transport copper to the TGN (i.e., behaves as a disease variant).

ATP7B-Arg⁸⁷⁵ Is Mislocalized Under Basal Conditions but Traffics Normally in Response to Copper. Normally, ATP7B resides in the TGN but traffics in response to changing copper levels. In elevated copper, ATP7B moves to vesicles to sequester excess copper, whereas copper depletion induces the return of ATP7B from vesicles to the TGN (11, 12). Therefore, the lack of copper delivery to the TGN could be attributable to either the retention of ATP7B-Arg⁸⁷⁵ in the endoplasmic reticulum (ER) or its inability to return to the TGN from vesicles. To discriminate between these scenarios, we characterized the ATP7B-Arg⁸⁷⁵ localization at different copper concentrations. (To minimize potential artifacts associated with protein overexpression, we used tet-regulated expression in Hek293Trex cells with 20 ng/mL doxycycline and expression time optimized before the experiments).

Control ATP7B-Gly⁸⁷⁵ showed expected TGN staining (Fig. 2A). In contrast, the ATP7B-Arg⁸⁷⁵ had a perinuclear ER-like pattern. Costaining with the ER marker calnexin and the TGN marker TGN38 confirmed that ATP7B-Arg⁸⁷⁵ was retained in the ER and was absent in the TGN (Fig. 2B). Unexpectedly, despite the loss of TGN targeting, the trafficking behavior of ATP7B-Arg⁸⁷⁵ appeared normal [i.e., in response to high (100 μ M) copper, ATP7B-Arg⁸⁷⁵ relocalized to vesicles]. Furthermore, once in

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¹A.G. and A.B. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: lutsenko@jhmi.edu.

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Fig. 1. Transport activity and expression of ATP7B-Arg⁸⁷⁵ and ATP7B-Gly⁸⁷⁵ in YSTT cells. (A) Tyrosinase assay under basal copper conditions shows pigment formation for ATP7B-Gly⁸⁷⁵ and no pigment for ATP7B-Arg⁸⁷⁵. (B) Immunostaining illustrates that both variants are expressed. (Scale bar: 14 μ m.)

vesicles, ATP7B-Arg⁸⁷⁵ could cycle back upon copper depletion with the copper chelator bathocuproine disulfonate (BCS) (Fig. 2C). Following this two-step (copper→BCS) treatment, ATP7B-Arg⁸⁷⁵ acquired proper TGN targeting instead of returning to its

Fig. 2. Intracellular localization of ATP7B-Arg⁸⁷⁵ is copper-dependent. (A) Immunostaining with anti-Flag antibody (green) illustrates the marked difference in the localization patterns of the Flag-tagged ATP7B-Arg⁸⁷⁵ (diffuse ER-like) and ATP7B-Gly⁸⁷⁵ (TGN-like) in basal medium. (B) (Upper) Costaining with the ER marker calnexin (red) and anti-Flag antibody (green) shows ER localization for ATP7B-Arg⁸⁷⁵. (Lower) No colocalization is observed with the TGN marker (red). CNX, calnexin. (C) (Left) Following treatment with 100 or 200 μ M copper (shown), ATP7B-Arg⁸⁷⁵ redistributes to vesicles. (Right) Subsequent treatment with 50 μ M BCS causes the relocation of ATP7B to the TGN. (Scale bar: 7 μ m.)

Fig. 3. Low copper (Cu) promotes ATP7B-Arg⁸⁷⁵ exit from the ER. Costaining with anti-Flag (green) and TGN38 (red) antibodies was performed following overnight incubation of Hek293Trex cells with 0–10 μ M copper. At 2 μ M copper, ATP7B-Arg⁸⁷⁵ is found predominantly in the TGN, whereas at 10 μ M copper, additional vesicular localization (arrows) becomes apparent. (Scale bar: 7 μ m.)

initial ER location. [Without copper pretreatment, BCS (bathocuproine disulfonic acid disodium salt) did not induce TGN targeting.] Thus, once ATP7B-Arg⁸⁷⁵ escaped the ER, it was targeted normally.

Physiological Copper Levels Promote ATP7B-Arg⁸⁷⁵ Exit from the ER and Restore Copper Delivery to the TGN. The trafficking of ATP7B-Arg⁸⁷⁵ from the ER to vesicles in response to high copper proceeds, presumably, via the TGN. This consideration led us to investigate whether low concentrations of copper would trigger ATP7B-Arg⁸⁷⁵ exit from the ER to the TGN. Overnight treatment of cells with 2 or 5 μ M copper resulted in loss of the ER pattern

Fig. 4. Relocalization to the TGN restores copper transport activity of ATP7B-Arg⁸⁷⁵. (A) Colocalization with TGN38 (red) staining confirms efficient targeting of Flag-ATP7B-Arg⁸⁷⁵ (green) to the TGN in the YSTT cells following treatment with 5 μ M copper. (Scale bar: 14 μ m.) (B) In the tyrosinase assay, pigment formation is observed in cells transfected with ATP7B-Arg⁸⁷⁵ and treated with 5 μ M copper; control cells treated with 5 μ M copper show no color.

Because 2–5 μM copper promoted normal TGN localization of ATP7B-Arg⁸⁷⁵, we were interested whether the same treatment would restore copper transport to the TGN. As in Hek293Trex cells, overnight treatment of YSTT cells with 0.5–10 μM copper resulted in the TGN targeting of Arg⁸⁷⁵-ATP7B (shown for 5 μM copper in Fig. 4A). The relocation was coupled with the appearance of black pigment, indicative of tyrosinase activation (Fig. 4B). This activation was not attributable to nonspecific copper entry into the TGN, because cells treated with the same copper concentrations showed no tyrosinase activity in the absence of ATP7B-Arg⁸⁷⁵. Thus, once the Arg⁸⁷⁵ variant reaches the TGN, it transports copper to the cuproenzyme(s).

negative effects because of its proximity to neighboring residues (Fig. 5A). To determine whether the Gly→Arg substitution alters the A-domain structure, we generated the recombinant A-domains with corresponding substitutions. Both domains showed robust expression in *Escherichia coli*, were soluble, and purified in milligram quantities, although the average yield of the Arg⁸⁷⁵ variant was lower (Fig. S3). The folding of the domains was examined by solution NMR at equal protein concentrations. The ¹H, ¹⁵N-HSQC (heteronuclear single quantum coherence) spectrum of the Gly⁸⁷⁵ A-domain showed excellent chemical shift dispersion, characteristic of a well-folded protein (Fig. 5B). In contrast, the Arg⁸⁷⁵ A-domain had poor signal dispersion indicative of the loss of structure. Thus, Arg⁸⁷⁵ promotes unfolding of the isolated A-domain. To evaluate the effect of Arg⁸⁷⁵ on the full-length ATP7B, we expressed ATP7B-Gly⁸⁷⁵ and ATP7B-Arg⁸⁷⁵ in HEK293Trex cells and compared protein amounts by Western blot analysis. In crude cell lysates, the ATP7B-Arg⁸⁷⁵ was consistently less abundant than the Gly variant. The difference became particularly pronounced after the isolation of microsomal membranes (Fig. 5C).

A-Domain Interacts with the N-Terminal Copper-Binding Domain. The A-domain does not bind copper; hence, to understand the effect of copper on the A-domain, we examined whether the A-domain interacted with the N-terminal copper-binding domain of ATP7B (*N*-ATP7B). By copurifying the recombinant *N*-ATP7B and the A-

Fig. 7. Copper increases ATP7B-Arg⁸⁷⁵ abundance and diminishes its post-ER degradation. (A) Western blots of membrane preparations from HEK293T cells transfected with Flag-ATP7B-Arg⁸⁷⁵ and treated overnight with or without 5 μM copper. (B) ATP7B-Arg⁸⁷⁵ was first targeted to the TGN by overnight treatment with copper; cells were then transferred to fresh medium with 5 μM copper or 50 μM copper chelator BCS, and protein levels were measured over time in the presence of CHX. Staining of Na,K-ATPase is used as a loading control.

was higher in the overloaded cells compared with cells in basal medium or cells treated with low copper (Fig. S6A), ATP7B-Arg⁸⁷⁵ was localized in the ER (Fig. S6B). Thus, bioavailable copper, rather than accumulated copper, rescues ATP7B-Arg⁸⁷⁵.

Chemotherapeutic Drug DDP Facilitates TGN Targeting of ATP7B-Arg⁸⁷⁵. Finally, we tested whether drugs that mimic the effect of copper on ATP7B can influence ATP7B-Arg⁸⁷⁵ localization and activity. We have previously found that a chemotherapeutic drug, DDP, binds to *N*-ATP7B (16, 17) and stabilizes the copper-bound-like state of ATP7B without competing with copper for transport sites (5). Therefore, we examined whether DDP would trigger ATP7B-Arg⁸⁷⁵ trafficking to the TGN and restore copper delivery to tyrosinase in the absence of copper additions.

Treatment with 10 μM DDP for 4 h induced ATP7B-Arg⁸⁷⁵ trafficking to the TGN (Fig. 8A) and activated tyrosinase (Fig. 8B). Fewer cells showed pigment formation, however, and the pigment color intensity was lower compared with cells treated with copper (Fig. 4B). To examine whether the lower transport activity was attributable to the low affinity of ATP7B-Arg⁸⁷⁵ for basal copper, cells were first treated with DDP to relocate ATP7B-Arg⁸⁷⁵ to the TGN and then briefly (for 15 min) exposed to copper to stimulate transport (without altering protein amounts). In this case, the pigment intensity and the number of cells showing pigment formation were significantly higher than in cells exposed only to DDP (4 h). This observation is consistent with ATP7B-Arg⁸⁷⁵ requiring extra copper for its transport activity. At the same time, the color intensity, although increased, still did not reach that of cells exposed to copper for longer times (4 h, when ATP7B is protected against degradation) (Fig. 8C). Thus, the full transport activity of ATP7B-Arg⁸⁷⁵ also requires protein stabilization by copper.

Discussion

The Arg⁸⁷⁵ variant of ATP7B was previously found to be a disease-causing mutation in patients with WD as well as a polymorphism in the normal population. We demonstrate that cellular localization, stability, and activity of ATP7B-Arg⁸⁷⁵ greatly depend on available copper. At a basal copper level, Arg⁸⁷⁵-ATP7B resides in the ER and shows no copper delivery to the TGN-located enzymes, whereas when more copper is available, it traffics to the TGN, restoring copper delivery. Thus, copper fluctuations may greatly influence the phenotype of this variant. In human populations, individuals heterozygous for WD mutations on a common Gly⁸⁷⁵ background typically do not show disease manifestations. Our results suggest that individuals with the same mutation on the Arg⁸⁷⁵ background may have the WD phenotype under certain conditions, such as copper deficiency. Additionally, patients homozygous for the WD mutation in combination with the Arg⁸⁷⁵ background may have a more severe disease phenotype or an earlier onset.

We demonstrate that the recombinant A-domain with Arg⁸⁷⁵ is unstable and unfolds during purification, likely because of the unfavorable effects of a bulky Arg side chain on neighboring residues. In the full-length ATP7B, interactions with other domains prevent complete protein unfolding (as evident from the preser-

Fig. 8. DDP partially mimics the effects of copper (Cu). (A) Colocalization of Flag-ATP7B-Arg⁸⁷⁵ (green) and TGN marker (red) in YSTT cells treated with 10 μM DDP for 4 h. (Scale bar: 7 μm.) (B) In the tyrosinase assay, pigment formation was observed in DDP-treated cells transfected with ATP7B-Arg⁸⁷⁵. (C) Comparison of the color intensity of pigment for cells transfected with ATP7B-Arg⁸⁷⁵ and treated with copper (15 min), DDP (4 h), DDP (4 h) plus copper (15 min), and copper (4 h). Pigment intensity was quantified using Image J software (National Institutes of Health); the effect of dual-DDP/copper treatment exceeds the additive.

vation of ATP7B trafficking and activity). Nevertheless, under basal conditions, imperfections of the ATP7B-Arg⁸⁷⁵ structure are detected by the ER quality control, causing protein retention. Defects in protein folding have been reported for numerous human disorders, and there is a significant need for identifying factors that can enhance protein folding and/or stability (18). We demonstrate that in the case of ATP7B-Arg⁸⁷⁵, the native ligand copper markedly improves the transporter's properties.

How does elevated copper correct the Arg⁸⁷⁵ phenotype? We observed that the Arg⁸⁷⁵ A-domain interacted with *N*-ATP7B and that copper binding to *N*-ATP7B enhanced this interaction. Although the structural basis for this increase is not yet known, higher stability of ATP7B-Arg⁸⁷⁵ in copper-treated cells suggests that interdomain interactions impart extra rigidity to the protein. In vitro, copper binding to copper-ATPases stabilizes a distinct protein conformation (5, 19). Thus, we speculate that copper-induced restriction of conformational mobility allows the ATP7B-Arg⁸⁷⁵ to exit from the ER, the initial key step in overcoming the disease phenotype. DDP, which binds to *N*-ATP7B and stabilizes ATP7B in a conformation resembling the copper-bound form (5), mimics the effects of copper.

In summary, our studies reveal how genetic and nongenetic factors can work together in determining the WD phenotype. DDP-mediated rescue of ATP7B-Arg⁸⁷⁵ encourages the search for drugs that stabilize ATP7B and improve protein targeting and function.

Materials and Methods

Additional details are provided in *SI Materials and Methods*.

Cell Lines and Microscopy. HEK293T cells were grown in DMEM supplemented with 10% (vol/vol) FBS, 15 $\mu\text{g}/\text{mL}$ blasticidin, and 100 $\mu\text{g}/\text{mL}$ zeocin; protein expression was induced with 20 ng/mL doxycycline. YSTT cells were maintained in DMEM with 10% (vol/vol) FBS, 200 $\mu\text{g}/\text{mL}$ G418, and 0.5 $\mu\text{g}/\text{mL}$ puromycin. The pcTYR plasmid was described earlier (10). For localization studies, the N-terminal FLAG tag was added to ATP7B by PCR and the construct was cloned into the pcDNA5 FRT/TO plasmid (Invitrogen). The 2623A $\rightarrow$ G substitution was introduced using a QuikChange protocol. The immunodetection of FLAG tag, calnexin, and TGN-38 was performed using commercial antibodies as described in *SI Materials and Methods*; images were acquired using confocal microscopy.

Copper, DDP, and Zinc Treatments. CuCl_2 (0.5–200 μM) was added to the growth medium for different time intervals; DDP (Sigma) was dissolved in DMSO and used at 10 μM for 4 h. Cells treated with an equal volume of DMSO were used as a control. Atomic absorption spectroscopy confirmed the lack of copper in the DDP solutions. ZnCl_2 (100 μM) was added for 4 h.

Studies of the A-Domain. The ATP7B fragment (residues 789–907) encoding the A-domain with Arg or Gly at position 875 was cloned into the pTXB1 vector and expressed in *E. coli*. Proteins were metabolically labeled with $^{15}\text{NH}_4\text{Cl}$; purified on chitin beads; cleaved using 2-mercaptoethane sulfonate

sodium (Sigma); and dialyzed into 50 mM Na-phosphate (pH 7.0), 50 mM arginine, and 50 mM glutamate. The ^1H , ^{15}N -HSQC spectra were recorded with 0.2 mM protein.

Tyrosinase Activation Assay. YSTT cells transfected with tyrosinase with or without ATP7B were incubated with or without copper/DDP (see above) and fixed for 30 s in cold acetone-methanol (1:1). L-DOPA was added as originally described (9), and formation of black pigment was detected by phase microscopy.

ATP7B Stability in Cells. HEK293T cells in 6-cm plates were transfected with 6 μg of ATP7B cDNAs and treated overnight with doxycycline (20 ng/mL) in basal medium. Cells were then transferred to fresh medium containing 50 μM CHX and incubated for 4 h with or without 5 μM copper. Microsomal fractions were isolated, and the protein amounts were analyzed by Western blot using the anti-Flag and anti-Na,K-ATPase antibodies.

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