Altered intracellular localization and valosin-containing protein (p97 VCP) interaction underlie ATP7A-related distal motor neuropathy

Ling Yi1, Anthony Donsante1, Marina L. Kennerson2, Julian F.B. Mercer3, James Y. Garbern4,† and Stephen G. Kaler1,*

1Unit on Human Copper Metabolism, Molecular Medicine Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, MD, USA, 2Northcott Neuroscience Laboratory, ANZAC Research Institute, University of Sydney, Concord, Australia, 3Centre for Cellular and Molecular Biology, School of Life and Environmental Sciences, Deakin University, Burwood, Australia and 4Department of Neurology and Center for Translational Neuromedicine, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA

Received October 21, 2011; Revised November 28, 2011; Accepted December 22, 2011

ATP7A is a P-type ATPase that regulates cellular copper homeostasis by activity at the trans-Golgi network (TGN) and plasma membrane (PM), with the location normally governed by intracellular copper concentration. Defects in ATP7A lead to Menkes disease or its milder variant, occipital horn syndrome or to a newly discovered condition, ATP7A-related distal motor neuropathy (DMN), for which the precise pathophysiology has been obscure. We investigated two ATP7A motor neuropathy mutations (T994I, P1386S) previously associated with abnormal intracellular trafficking. In the patients’ fibroblasts, total internal reflection fluorescence microscopy indicated a shift in steady-state equilibrium of ATP7AT994I and ATP7AP1386S, with exaggerated PM localization. Transfection of Hek293T cells and NSC-34 motor neurons with the mutant alleles tagged with the Venus fluorescent protein also revealed excess PM localization. Endocytic retrieval of the mutant alleles from the PM to the TGN was impaired. Immunoprecipitation assays revealed an abnormal interaction between ATP7AT994I and p97/VCP, an ubiquitin-selective chaperone which is mutated in two autosomal dominant forms of motor neuron disease: amyotrophic lateral sclerosis and inclusion body myopathy with early-onset Paget disease and fronto-temporal dementia. Small-interfering RNA (SiRNA) knockdown of p97/VCP corrected ATP7AT994I mislocalization. Flow cytometry documented that non-permeabilized ATP7AP1386S fibroblasts bound a carboxyl-terminal ATP7A antibody, consistent with relocation of the ATP7A di-leucine endocytic retrieval signal to the extracellular surface and partially destabilized insertion of the eighth transmembrane helix. Our findings illuminate the mechanisms underlying ATP7A-related DMN and establish a link between p97/VCP and genetically distinct forms of motor neuron degeneration.

INTRODUCTION

ATP7A is a copper-transporting ATPase that helps regulate and control cellular copper homeostasis (1). Defects in ATP7A lead to Menkes disease, or its allelic variants occipital horn syndrome (OHS), and isolated distal motor neuropathy (DMN), a recently identified condition (2). Whereas Menkes disease and OHS share specific clinical and biochemical abnormalities (3), subjects with ATP7A-related DMN manifest normal serum copper, normal copper enzyme activities, normal renal tubular function and no central nervous system or connective tissue abnormalities (2). Conversely, subjects with Menkes disease and OHS are not known to develop motor neuron dysfunction (although formal neurophysiological studies have not yet been reported). These cumulative findings imply that the mechanism(s) of disease in the new allelic variant affecting purely motor neurons could be distinctly different than for Menkes and OHS (1,2).

*To whom correspondence should be addressed at: National Institutes of Health, Building 10, Room 10N313, 10 Center Drive MSC 1853, Bethesda, MD 20892-1853, USA. Tel: +1 3014516034; Fax: +1 3014808657; Email: kalers@mail.nih.gov
†Deceased.

Published by Oxford University Press 2011.
Moreover, discovery of this new allelic variant disclosed that ATP7A, and copper metabolism in general, plays a crucial role in motor neurons which remain to be fully illuminated. ATP7A is expressed ubiquitously, resides in the trans-Golgi network (TGN) compartment of cells and transports cytoplasmic copper to that compartment for incorporation into copper enzymes. ATP7A relocates to the plasma membrane (PM) of cells in response to increased intracellular concentration of this metal (4), where it mediates copper exodus from the cell, and recycles back to the TGN, possibly via clathrin-mediated endocytosis (5). Many of the specific molecular domains responsible for the intracellular trafficking of ATP7A, and their effects, have been identified (1).

Some axonal neuropathies that are clinically similar to ATP7A-related DMN, such as Charcot–Marie–Tooth, type 2 disease, are caused by mutations in genes associated with mitochondrial function, axonal transport or endosomal trafficking (6). Other syndromes featuring motor neuron degeneration have been associated with mutations in valosin-containing protein (p97/VCP), a hexameric ATPase involved in multiple cellular functions, including vesicular trafficking and degradation of proteins by the ubiquitin (Ub)-proteasome system (UPS) (7,8).

Copper deficiency myelopathy is a well-known clinical entity, reflecting the relationship between acquired copper deficiency from various causes and mixed sensory and motor peripheral neuropathy (9–15). Patients with Menkes disease have impaired absorption of copper which leads to systemic copper deficiency from loss-of-function ATP7A mutations, whereas ATP7A mutations that cause isolated DMN have not been associated with low copper levels in blood (2).

In this study, we applied clinical, biochemical, cellular and molecular approaches to evaluate mechanisms underlying ATP7A-related DMN and begin to elucidate the normal function of ATP7A in motor neurons.

**RESULTS**

**Patient evaluations**

Three patients with classic Menkes disease (age range 17–23 months) and three patients with OHS (ages 33 months, 19 years and 31 years) underwent clinical and electrophysiological evaluation for evidence of peripheral neuropathy. There was no suggestion on physical examinations or in nerve conduction studies of DMN in any of these individuals (Supplementary Material, Table S1). These results in subjects with diverse missense or splice junction ATP7A mutations contrasted with the distinctly abnormal peripheral nervous system findings in previously studied subjects from the two families with ATP7A-related DMN (2). Clinical examinations of the latter patients, whose initial neuropathic symptoms occurred between age 2 and 61 years, were notable for distal muscle weakness and decreased deep tendon
reflexes. Nerve conduction studies often showed decreased distal motor action potential amplitudes, indicative of axonal dysfunction. In the family in which the P1386S mutation segregated, affected subjects frequently showed clinical and electrophysiological evidence of both sensory and motor neuron dysfunction (2).

Altered intracellular localization of mutant ATP7A alleles causing motor neuronopathy

Previous characterization of the T994I and P1386S mutant ATP7A alleles indicated delayed trafficking from the TGN in response to elevated copper concentrations in fibroblasts cultured at subnormal (30°C) temperature (2). The abnormal trafficking at 30°C raised the possibility that these variants might represent a new class of ATP7A temperature-sensitive mutations. However, yeast complementation assays to assess residual copper transport capacity in S. cerevisiae complementation assays (Fig. 1B). However, we found consistent evidence of diffuse ATP7A signal not localized to the TGN in ATP7AT994I and ATP7AP1386S fibroblasts cultured at normal temperature (37°C) (Fig. 2), and sought to determine the precise location(s). Employing confocal microscopy and immunohistochemical analyses with organellar-specific markers, we found that the mutant ATP7As did not clearly localize to the endoplasmic reticulum, early or late endosomes, lysosomes or endocytic vesicles (Fig. 3, Supplementary Material, Fig. S1). However, total internal reflection fluorescence (TIRF) microscopy indicated a shift in the steady-state equilibrium of ATP7AT994I and ATP7AP1386S with increased localization in the vicinity of the PM (Fig. 4). Transfection of Hek293T and undifferentiated NSC-34 motor neuron cells with enhanced yellow fluorescent protein (EYFP) Venus-tagged mutant alleles suggested a shift in the steady-state equilibrium of ATP7AT994I and ATP7AP1386S to excess PM localization relative to normal, under basal copper concentrations (0.5 μM Cu) (Fig. 5C, D, G, H). Approximately 20–30% of cells transfected with the mutant alleles showed TGN localization, in comparison to 85–90% of cells transfected with wild-type ATP7A. This pattern was reminiscent of the wild-type ATP7A signal under elevated copper exposure (200 μM Cu) (Fig. 5B and F). In differentiated NSC-34 cells (Fig. 5, NSC34-D, lower panel), neuritic projections, which stained positive for the axonal marker Tau-1 (Supplementary Material, Fig. S2), demonstrated wild-type ATP7A signal along their full length (Fig. 5I), with localization to the axonal membrane following addition of 200 μM copper to the culture medium (Fig. 5J). In contrast, the projections from differentiated NSC-34 cells transfected with ATP7AT994I and ATP7AP1386S showed signal predominantly at the axonal membrane under basal copper concentrations (0.5 μM Cu) (Fig. 5K and L).

Figure 2. Fibroblasts derived from individuals with ATP7A-related DMN show abnormal localization of ATP7A. (A) Color-merged images of normal human fibroblasts (CRL2076), and from patients with ATP7AT994I and ATP7AP1386S. Cells were co-stained with antibodies against ATP7A (green) and TGN64, a trans-Golgi marker (red), and with DAPI (4′,6 diamidino-2-phenylindole, dihydrochloride) nuclear counterstain (blue). The normal cells show co-localization (yellow) of ATP7A and TGN46, whereas the mutant fibroblasts show reduced trans-Golgi localization and more diffuse ATP7A signal (green) throughout the cells. (B) Epifluorescence images of human fibroblasts stained with a carboxyl-terminal antibody to ATP7A. Note tight perinuclear signal in the wild-type cells compared with those in the affected patients’ cells, in which perinuclear staining is less discrete. Scale bars = 10 μm.
Delayed endocytic retrieval of ATP7A<sup>T994I</sup> and ATP7A<sup>P1386S</sup>

To explore the basis for increased PM localization, we evaluated endocytic retrieval of ATP7A to the TGN in transfected Hek293T cells (Fig. 6). As previously described, ATP7A ordinarily resides in the TGN and moves to the PM in response to high intracellular copper concentrations (4). Lowering intracellular copper with the copper chelator bathocuproine disulfonate (BCS) induces recycling of ATP7A from the PM back to the TGN. Thus, in order to provide an appropriate wild-type control for assessment of endocytic retrieval, we cultured Hek293T cells in high copper (200 μM) for 3 h. This treatment located wild-type ATP7A predominantly at the PM (Fig. 6A), a distribution approximating that of ATP7A<sup>T994I</sup> and ATP7A<sup>P1386S</sup> under basal copper concentrations (0.5 μM, Fig. 6Ab and c). We then concurrently treated all the transfected Hek293T cells with BCS, which, as expected, resulted in retrieval of Venus-tagged wild-type ATP7A from the PM toward the TGN (Fig. 6Ad). This fluorescent signal represented only ATP7A originally at the PM, since we co-treated all cells with cycloheximide (CHX) to eliminate de novo protein synthesis. In contrast, ATP7A<sup>T994I</sup> and ATP7A<sup>P1386S</sup> remained largely at the PM after BCS/CHX treatment (Fig. 6Ae, f and B), indicating blocked retrograde trafficking of these mutant alleles in response to copper washout. The excess PM localization of the ATP7A<sup>T994I</sup> and ATP7A<sup>P1386S</sup> mutant alleles was not due to aberrant interaction with lipid rafts, since sucrose gradient
Figure 4. TIRF microscopy indicates a shift in the steady-state equilibrium of ATP7A T994I and ATP7AP1386S, producing increased PM localization. (A) Under normal copper concentrations (top panel), wild-type fibroblasts display minimal quantities of ATP7A signal at the PM, whereas increasing the copper concentration in the media to 200 μM for 3 h (bottom panel) triggers increased accumulation, as expected. (B) Wide field epifluorescence (top panels) and TIRF (bottom panels) microscopic images of wild-type and mutant fibroblasts under basal copper conditions. Note more diffuse signal in the mutant cell wide field views and increased signal in the mutant cell TIRF images, the latter indicating excess localization at or in the vicinity of the PM relative to wild-type cells. Scale bar = 10 μm.

Figure 5. Transfection of Hek293T cells, undifferentiated and differentiated NSC-34 motor neurons with ATP7A T994I and ATP7A P1386S reveals a shift in the normal protein localization pattern. (A) In Hek293T cells (top panel) transfected with Venus-tagged plasmids harboring the respective ATP7A alleles, the mutant proteins demonstrate excessive PM localization under basal copper concentrations (C and D), a pattern reminiscent of the wild-type protein under increased copper exposure (B). A minority of cells transfected with mutant alleles show perinuclear (TGN) signal (arrows). In NSC-34 cells, the cell bodies and axons (induced under culture conditions that promote motor neuron differentiation) display excess PM localization when transfected with the mutant alleles (G, H, K, L). The axonal location of the Venus-tagged wild-type ATP7A in these cells is dependent on the media concentration of copper, being detected in the lumen of the axon under basal copper conditions (I), and at the axonal membrane in response to higher copper (J). Scale bar, 10 μm.
experiments documented co-migration with transferrin receptor, a non-raft membrane protein (Supplementary Material, Fig. S3).

Abnormal interaction of ATP7A<sup>T994I</sup> with p97/VCP
Since abnormal protein–protein interactions have been implicated in numerous other inherited motor neuropathies (16–20), we performed immunoprecipitation assays using total cell lysates from Hek293T cells transfected with the wild-type and two mutant ATP7A alleles (Fig. 7). The single peptide band that uniquely co-precipitated with the T994I mutant allele (Fig. 7A) was excised from a Coomassie brilliant blue-stained gel, sequenced for amino acid content and identified as a 100% match to valosin-containing protein 97 (p97/VCP). The interaction between p97/VCP and ATP7A<sup>T994I</sup> was confirmed by repeat immunoprecipitation and western blotting (Fig. 7B). In the converse experiment, we documented that ATP7A<sup>T994I</sup>
was selectively immunoprecipitated by p97/VCP (Fig. 7C). A distinct though less intense interaction with p97/VCP was evident for both wild-type ATP7A and ATP7A P1386S (Fig. 7B). Valosin-containing protein is among 1000 proteins in the Golgi proteome (21) and also localizes to the endoplasmic reticulum and nucleus (22). p97/VCP functions include vesicular trafficking (fusion of transport vesicles with target membranes), involvement in the UPS, endoplasmic reticulum-associated degradation (ERAD) and autophagy (22,23). Mutations in p97/VCP cause two autosomal dominant motor neuron diseases: inclusion body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD), and familial amyotrophic lateral sclerosis (ALS) (7,8,22,23). The coincident occurrence of motor neuron dysfunction and myopathy in IBMPFD has been carefully discussed (8).

The interaction between ATP7A T994I and p97/VCP was not due to proteosomal degradation or ERAD, since western blots did not show excess staining with an anti-ubiquitin antibody (data not shown). Furthermore, ATP7A T994I fibroblasts and transfected Hek293T cells did not manifest increased expression of BiP or the XBP-1 splice variant, hallmarks of the mammalian misfolded protein response (24) (data not shown).

Since p97/VCP is known to interact with ubiquitin-binding (UBX) regions (25), we searched for potential sequences within ATP7A that might mimic these domains, and identified two regions of minor homology, one in a lumenal loop of ATP7A adjacent to the T994I mutation in the sixth transmembrane segment, and one in the cytoplasmic portion near the conserved phosphatase motif of ATP7A (Supplementary Material, Fig. S4). Both regions feature a phenylalanine–proline (FP) dipeptide, a motif found in UBX domains and recently proposed to interact with the N domain of p97/VCP, based on the crystal structural analysis (26). The cytosolic region of homology overlaps the ATP7A actuator (A) domain, which harbors a phosphatase motif important for ATP7A dephosphorylation during the E2 stage of the Post-Albers catalytic cycle (27). Binding of p97/VCP at this site could explain PM accumulation of ATP7A T994I since mutations within or near the ATP7A phosphatase motif are known to cause sustained phosphorylation; catalytic hyperphosphorylation is associated with increased ATP7A targeting to and prolonged retention at the PM (28,29).

To evaluate the possible role of aberrant allosteric p97/VCP interaction in ATP7A T994I intracellular mislocalization, we performed small-interfering RNA (siRNA) knockdown of p97/VCP in transfected Hek293T cells. We hypothesized that knockdown would reduce interaction with p97/VCP and correct ATP7A T994I mislocalization. As predicted, cells in

Figure 7. Immunoprecipitation assays identify p97/VCP as an interacting protein with ATP7A T994I. (A) Coomassie brilliant blue-stained gel of proteins immunoprecipitated by a polyclonal anti-GFP antibody following transfection of Hek293T cells with the respective Venus-tagged ATP7A alleles. The larger arrow (top) indicates the Venus + ATP7A fusion protein of approximate molecular mass 206 kDa. The smaller arrow highlights a thin band, unique to ATP7A T994I, which was identified on amino acid sequencing as valosin-containing protein (p97/VCP). (B) Western blot analyses of subsequent immunoprecipitation assays to confirm the interaction of p97/VCP with ATP7A. Following transfection of Hek293T cells with the respective Venus-tagged ATP7A cDNAs, total cell protein was immunoprecipitated with a polyclonal anti-GFP antibody, denatured and separated by electrophoresis, transferred to PVDF membrane and probed with monoclonal antibodies against GFP (top two panels) or p97/VCP (bottom two panels). Small quantities of p97/VCP are detected in association with wild-type ATP7A and ATP7A P1386S, and a more prominent (10-fold higher by densitometric quantification) signal is evident in association with ATP7A T994I (third panel). The total lysate (TL) blot confirmed equivalent sample loading. (C) Following co-transfection of Hek293T cells with FLAG-tagged p97/VCP and the respective Venus-tagged ATP7A cDNAs, total cell protein was immunoprecipitated with anti-FLAG M2 affinity gel, denatured and separated by electrophoresis, transferred to PVDF membrane and probed with monoclonal anti-FLAG (top panel) or anti-GFP antibodies (two bottom panels). Easily visible signal is seen in association with ATP7A T994I (middle panel). The ATP7A-Venus signal detected in the total lysates (TL) implies equivalent expression of the wild-type ATP7A, ATP7A T994I and ATP7A P1386S alleles.
which p97/VCP was decreased to \( \sim 40\% \) of the wild-type (Fig. 8A and B) showed a significant increase in trans-Golgi localization (Fig. 8C and D).

**Destabilized eighth transmembrane helix insertion of ATP7A<sup>P1386S</sup>**

Since the interaction of p97/VCP with ATP7A<sup>P1386S</sup> was no different from the wild-type (Fig. 7B and C) and siRNA knockdown of p97/VCP did not significantly correct the P1386S cell biological phenotype (Fig. 8C and D), we considered alternative mechanisms for excess PM localization of ATP7A<sup>P1386S</sup>. The recently identified crystal structure of CopA, a bacterial homolog of ATP7A, indicated that proline 1386 is positioned precisely at the entry to the membrane (30) (Supplementary Material, Fig. S4), rather than in the center of the fourth lumenal loop, as previously thought, based on hydropathy plot predictions (31). We designed fluorescence-activated cell sorting (FACS) experiments to evaluate whether substitution of serine for proline 1386 might have a ‘helix-breaker’ effect and destabilize insertion of the eighth transmembrane domain of the molecule into the PM, as has been reported for numerous other large transmembrane proteins (32,33). This phenomenon could partly explain preferential accumulation of ATP7A<sup>P1386S</sup> at the PM, since it would result in relocation of the di-leucine signal known to mediate ATP7A endocytic retrieval (34,35) from the cytosolic to non-cytosolic protein face. Flow cytometry experiments indicated that mean fluorescence intensity from non-permeabilized fibroblasts stained with a carboxyl-terminal antibody (which includes the di-leucine signal) was significantly higher in ATP7A<sup>P1386S</sup> patient fibroblasts (Fig. 9A and B). This result suggested repositioning of the di-leucine motif and possibly the entire eighth transmembrane segment, to the extracellular surface of the PM in some fraction of the mutant molecules (Fig. 9C). This effect would likely completely abrogate copper release, since a methionine and a serine residue within this membrane segment normally coordinate copper binding (36). Thus, destabilized eighth transmembrane helix insertion is unlikely to represent the sole basis for retention of ATP7A<sup>P1386S</sup> at the PM. Our yeast complementation studies (Fig. 1B), and the affected patients’ clinical and biochemical phenotypes (2), further support this interpretation.

**DISCUSSION**

The present findings advance our understanding of the mechanisms that underlie ATP7A-related DMN. Prior
Figure 9. FACS suggests that the carboxyl-terminal tail of ATP7A\textsuperscript{P1386S} is partially outside the PM. (A) Mean fluorescence intensity measured by flow cytometry of non-permeabilized fibroblasts exposed to a carboxyl-terminal antibody against ATP7A that includes the di-leucine endocytic retrieval signal. Fluorescent signal from this antibody was detected predominantly in ATP7A\textsuperscript{P1386S} patient fibroblasts, consistent with positioning of the di-leucine motif (and possibly the entire eighth transmembrane segment) on the extracellular face of the PM. A well-characterized normal fibroblast cell line (CRL 2076) was used as control, in addition to ATP7A\textsuperscript{C994I} patient fibroblasts. (B) Mean fluorescence intensity (triplicate measures) from triplicate flow cytometry experiments. Bars represent ± standard error. (C) The topological model of wild-type ATP7A in relation to the TGN and PM, illustrating partially destabilized eighth transmembrane helix insertion in ATP7A\textsuperscript{P1386S}. The dashed line denotes the proper PM insertion of the eighth transmembrane segment. The epitope for the ATP7A antibody used is shown in red, and the X indicates the putative block in endocytic return of the mutant molecule to the TGN (also see Fig. 6).

Figure 10. Structural model of ATP7A generated by Polyview-3D and based on the crystal structure of CopA, a prokaryotic homolog (30), reveals proximity of the two amino acid residues (lavendar) associated with DMN. (A) Jmol orientation: dX = 45°, dY = 45°, dZ = 45° (B) Jmol orientation: dX = 90°, dY = 0°, dZ = 90° (C) Jmol orientation: dX = 0°, dY = 0°, dZ = 0°.
characterization of the associated clinical and biochemical phenotypes of this condition suggested clear distinctions from the pathophysiology of Menkes disease and OHS (1–3). Individuals with ATP7A-related DMN manifested no central neurological problems, and had no clinical or biochemical findings similar to those observed in patients with Menkes disease or OHS. Specifically, no patients with ATP7A-related DMN examined to date have shown hair, skin or joint abnormalities, low serum copper, abnormal plasma catecholamine levels or renal tubular dysfunction, all of which are considered phenotypic hallmarks of mutations at the ATP7A locus (1). Conversely, three patients with Menkes disease and three individuals with OHS examined showed no clinical or significant electrophysiological evidence of motor neuron dysfunction (Supplementary Material, Table S1). The individuals with OHS included the first patient in whom the condition was molecularly defined (3) who is now 31 years old, and an unrelated 19 years old (37). At these ages, it would be expected for ATP7A-related DMN to be clinically manifest, if a common pathogenetic mechanism were involved. Thus, ATP7A<sup>T994I</sup> and ATP7A<sup>P1386S</sup> do not appear to affect global copper metabolism as is evident for other ATP7A molecular defects (1,3,38). Yet these mutations, in the sixth and eighth transmembrane segments, respectively, demonstrate a specific effect on motor neuron function. Of potential relevance in this regard is the close alignment of the T994 and P1386 residues in an ATP7A model (Fig. 10) based on the crystal structure of CopA (30).

Here we show that p97/VCP interacts strongly with ATP7A<sup>T994I</sup>, linking the new ATP7A motor neuron phenotype with autosomal dominant forms of motor neuron degeneration, specifically IBMPFD and ALS (7,8,22,23). Mutations in p97/VCP have been implicated in both these latter conditions. The abnormal interaction of ATP7A<sup>T994I</sup> with p97/VCP may reduce the pool of active p97/VCP available for its normal cellular function. Alternatively, aberrant p97/VCP-mediated vesicular trafficking or endosomal sorting of ATP7A<sup>T994I</sup> may be a crucial consequence (23). The absence of obvious protein aggregates or TDP43 pathology associated with ATP7A<sup>T994I</sup> (data not shown) implies that p97/VCP loss of function underlies this motor neuron degeneration rather than a toxic gain-of-function (39). Further investigations are needed to clarify the precise mechanism(s) through which p97/VCP perturbs motor neuron function in these unrelated disorders.

In the context of ATP7A<sup>T994I</sup>, p97/VCP may function as an allosteric inhibitor obstructing the phosphatase domain of ATP7A (Supplementary Material, Fig. S4), and extending the duration of the ATPase catalytic cycle phosphorylation (27) without entirely abrogating copper transport (Fig. 1B). The effect may delay dephosphorylation and cause retention at the PM, as previously described for hyperphosphorylated ATP7As (28,29).

A possible clue to the mechanism of impaired motor neuron function mediated by ATP7A<sup>P1386S</sup> was provided by previous case reports in which transient copper deficiency induced a mixed motor and sensory axonal neuropathy with clinical and electrophysiological findings highly similar to those in patients with ATP7A<sup>P1386S</sup>. These reports (9–15) and others document the exquisite sensitivity of motor and sensory neurons to copper deficiency of varied etiology. We hypothesize that preferential PM localization of ATP7A<sup>P1386S</sup> as shown by TIRF imaging and mammalian cell transfections produced a gradual depletion of axonal copper in vivo, since the capacity to pump copper is impaired only subtly by this mutation (Fig. 1B). Non-catalytic phosphorylation induced by the P1386S substitution, particularly in the serine-rich carboxyl-terminal region of ATP7A (40), is one consideration since increased phosphorylation of specific residues could enhance trafficking of ATP7A<sup>P1386S</sup> to the PM and mediate copper egress even under conditions of low or normal axonal copper concentrations. Formal tests using a small molecule fluorophore such as Coppersensor-3 (CS3) capable of imaging labile copper pools (41), may be useful for documenting the effect of ATP7A<sup>P1386S</sup> on axonal copper content and distribution in transfected neuronal cells or primary motor neurons. The pure motor phenotype in the T994I family (2,42) rather than the mixed motor and sensory phenotype manifested in P1386S individuals (2) suggests that axonal copper deficiency may be a causative mechanism only for the latter mutation.

The shift to excess PM localization that we delineate here for both ATP7A<sup>T994I</sup> and ATP7A<sup>P1386S</sup> is not inconsistent with their delayed movement from the TGN in response to copper, a trafficking abnormality previously reported for these alleles (2). The slower response can be explained in one case by the presence of an abnormal ligand, p97/VCP, in association with ATP7A<sup>T994I</sup> (Fig. 7), and by trapping of the carboxyl terminal tail of some ATP7A<sup>P1386S</sup> molecules within the TGN lumen. The concept of impaired or destabilized transmembrane helix insertion is well-established for other membrane proteins, and felt to contribute to numerous disease conditions, including juvenile myoclonic epilepsy, X-linked Charcot–Marie–Tooth disease, diabetes insipidus, retinitis pigmentosa, cystic fibrosis, severe myoclonic epilepsy of infancy and Best macular dystrophy (32,33).

The precise fashion in which ATP7A is normally recycled from the PM to the TGN has not been established. Evidence for clathrin-dependent, clathrin-independent and caveolin-independent endocytosis has been reported (5,43). The presence of a di-leucine sorting signal in the carboxyl tail of multi-spanning transmembrane proteins (e.g. DKHS in ATP7A) typically denotes a capacity to bind adaptor protein/ clathrin complexes, which in turn mediate rapid internalization and targeting to early endosomes (44). ATP7A mutations that alter the di-leucine signal (34,35) or which disturb dephosphorylation of the ATPase (1,28,29) may result in excess retention of ATP7A at the PM.

Our results offer further insight on the role of ATP7A in motor neuron biology. Various copper enzymes, copper transporters and copper chaperones are expressed in mouse spinal cord (45). Thus, copper clearly seems to be required for normal mammalian motor neuron function. Based on the results reported here, we postulate that ATP7A normally traffics down axons and mediates copper release from the axonal membrane of motor neurons (Fig. S1 and J). The possibility that this process is dependent on neuronal activation, calcium release or a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors as suggested for other neuronal cell populations (46–48), will require additional experimentation to document.
Finally, our findings provide a window into possible treatment approaches for symptomatic as well as pre-symptomatic individuals with ATP7AT994I or ATP7AP1386S. In the case of ATP7AT994I, a motor neuron-directed viral gene therapy to add p97/VCP, or genome editing by zinc finger nucleases (49) to correct the ATP7A alteration represent potential therapeutic considerations. Copper replacement to attain serum levels near the upper limit of normal (125–150 μg/dl), or treatment with copper ionophores (50) that might enhance delivery to motor neurons could counter the axonal copper deficiency we postulate sustained PM localization of ATP7AP1386S induces. Knock-in murine models of ATP7AT994I and ATP7AP1386S will be useful for exploring the best option(s) for treatment.

MATERIALS AND METHODS

Subjects

All patients and patient specimens were studied under protocols approved by the NICHD or NINDS Institutional Review Board (IRB) and written informed consent was obtained from adult patients or minor patients' parents. Nerve conduction studies were performed at the NIH Clinical Center in Bethesda, MD, USA, with one exception.

Cell culture

Human fibroblasts were obtained from affected patients by skin punch biopsy under sterile conditions, or from apparently healthy individual cell lines GM03440 and CRLL-2076 from the Coriell Institute for Medical Research (Camden, NJ, USA) or ATCC (Manassas, VA, USA), respectively. Fibroblasts, Hek293T cells and NSC-34 neuronal cells were cultured in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal calf serum, antibiotics and l-glutamine under standard sterile culture conditions in a 5% CO2 incubator at 37°C. NSC-34 cells were also cultured in differentiation medium consisting of high-glucose DMEM/Ham’s F-12 (1:1) with 1% fetal calf serum and 1% non-essential amino acids for 6 days before transfections.

Cell transfections and confocal microscopy

Full-length ATP7A and human p97 VCP cDNAs were constructed by reverse transcription polymerase chain reaction using total RNA as template. Site-directed mutagenesis was used to generate the respective ATP7A mutant alleles. After sequence fidelity was confirmed, the cDNAs were inserted between the SalI and ApaI sites of pVenus-C1. The human p97/VCP cDNA was inserted between the BglII and KpnI sites of pFLAG-CMV-5.1 (Sigma). The constructs were used to transfect Hek293T cells, NSC-34 cells and differentiated NSC-34 motor neurons. Transfections were mediated by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions and were performed in triplicate. The following primary antibodies were used: sheep polyclonal TGN46 for TGN (Novus; used at 1:200); rabbit polyclonal anti-GRP94 for endoplasmic reticulum (Abcam; used at 1:200); mouse monoclonal anti-EEA1 for early endosomes (BD Biosciences; used at 1:250); mouse monoclonal anti-CI-M6PR for late endosomes (Abcam; used at 1:200); mouse monoclonal anti-LAMP1 for lysosomes (Santa Cruz; used at 1:200). Secondary antibodies used were goat anti-mouse or goat anti-rabbit IgG coupled to Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen; used at 1:4000). Wild-type- and ATP7A-transfected cells were examined by a confocal microscope (Zeiss) and images captured using META software. For experiments in which ATP7A intracellular localization was quantitated, details on cell scoring are provided below.

Western blotting

Total protein was isolated from cell supernatants after lysis and centrifugation, as described above, denatured by adding 5× loading buffer with 5% β-mercaptoethanol (Quality Biological Inc.) and heating at 50°C for 10 min. Samples (40 μg total protein) were electrophoresed through 4–12% NOVEX Tris-Glycine sodium dodecyl sulfate (SDS) polyacrylamide (Invitrogen), and transferred to polyvinylidene fluoride membranes. The membranes were incubated at 4°C overnight in Tris-buffered saline blocking buffer (0.9% (v/v) NaCl, 20 mM Tris/HCl, pH 7.5, 0.5% SDS (v/v), 0.1% Tween 20 (v/v) containing 5% (w/v) non-fat milk. Blots were washed with tris-buffered saline, and then incubated for 3 h with a 1:1000 dilution of rabbit anti-ATP7A antibody of known high-specificity (38), anti-β-actin (Abcam), anti-p97/VCP (BioLegend), anti-FLAG (Sigma) or anti-GFP (Clontech) antibodies. After washing, membranes were incubated with anti-rabbit IgG horseradish peroxidase conjugate (1:2000, Santa Cruz Biotechnology) for 1 h at room temperature, washed and developed using SuperSignal West Pico Luminol/Enhancer Solution (Pierce), according to the manufacturer’s instructions.

Yeast complementation assays

The respective cDNA constructs were generated and cloned into pYES6/CT (Invitrogen) plasmids, which were used to transform the Saccharomyces cerevisiae copper transport mutant, ccc2Δ, as previously described (1,38). The transformed and mock-transformed yeast strains were cultured in copper/iron-limited solid or liquid media, as previously described (2). Growth experiments were performed in sextuplicate and the means and standard deviations calculated for the individual strains.

TIRF microscopy

Primary fibroblasts were grown in four-well chambers, in an incubator at 37°C with 5% CO2. Cells were washed once with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 10 min, then permeabilized with 0.1% Triton X-100/PBS at RT for 10 min, blocked with 10% goat serum for 1 h and probed with an anti-ATP7A rabbit antibody (1:4000) overnight. After washing twice with PBS, 10% goat serum in PBS containing Alexa fluor 488-conjugated anti-rabbit IgG was applied at RT for 30 min. The buffer was then removed, the cells washed twice with PBS and images collected with an Olympus TIRF microscope at 488 objective.
Endocytic retrieval studies

Hek293T cells were transfected with WT ATP7A-EYFP (‘Venus’ enhanced yellow fluorescent protein) as described above, and pulsed with 200 μM Cu for 3 h in order to drive ATP7A to the PM and replicate the primary location of ATP7A in cells transfected with T994I and P1386S under basal copper conditions. The cell cultures were then simultaneously exposed to 50 μM BCS (to remove Cu) and 10 mM CHX (to inhibit protein synthesis) for 4 h prior to confocal imaging. For quantitation of ATP7A intracellular localization (Fig. 6B), 150–200 cells from five to eight independent confocal images per transfection were scored, and the sample means and standard deviations calculated.

Immunoprecipitation assays and amino acid sequence analyses

Hek293T cells were transfected with the respective EYFP-tagged ATP7A (Venus tag) alleles. Twenty-four hours post-transfection, the cells were rinsed thrice with PBS, and lysed in lysis buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 10% glycerol, protein inhibitor mixture (Roche)] on ice for 20 min. The lysate was centrifuged at 10 000g for 10 min, and supernatants used for immunoprecipitation with rabbit anti-GFP antibody (Clontech) and protein G agarose beads (Thermo Scientific) or anti-FLAG M2 Affinity Gel (Sigma). Immunoprecipitates were denatured with 2% SDS and electrophoresed through a 10% polyacrylamide gel and stained with Coomassie brilliant blue. Selected bands were excised and were ‘in-gel’ digested with trypsin, and the resulting peptides were extracted. Peptides were analyzed both by MALDI TOF/TOF using an ABI 4800 Proteomics Analyzer (Applied Biosystems) and by combined liquid chromatography/tandem mass spectrometry (LC/ESI/MS/MS) using a LCQ DECA ion trap mass spectrometer (Thermo Fischer). Tryptic peptides were separated by reverse phase chromatography and electrosprayed directly into the sampling orifice of the mass spectrometer. MS/MS spectra were collected in a data-dependent manner, with up to three of the most intense ions in each full MS scan being subjected to isolation and fragmentation. MS/MS spectra were extracted as data files using default parameters with BioWorks v2.0 (Thermo Fisher). Peptide and protein identifications were validated using Scaffold v2.2 (Proteome Software, Portland, OR, USA). The cutoff for peptide identification was set at >95.0% probability and protein identification at >99% probability with two or more identified peptides.

siRNA assays

RNA-mediated interference of p97/VCP was performed by using siRNA duplexes purchased from Dharmacon:

5′-GCAUGUGGGUGGUGACUUA-3′, 5′-CAAAUUGGCU GGUGAGUCU-3′, 5′-CCUGAUUGUCUGAGCUGUA-3′ and 5′-GUAACUCUCUUCCGAGGUAUA-3′. SiRNA oligonucleotides (100 pmoles human VCP or non-targeting siRNA) were diluted in 50 μl Opti-MEM medium and mixed gently with Lipofectamine 2000. One hundred microliter of the siRNA-Lipofectamine complex was then added to Hek293T cells in six-well plates and incubated at 37 °C CO2 for 48 h. The cells were then passaged into new six-well plates (2×105 cells/well) or 2×4-well chambers (0.75×105 cells/well), incubated for 16 h and transfected with the respective ATP7A cDNA constructs (wild-type, T994I, P1386S). The cells were incubated at 37 °C in 5% CO2 for 24 h, and images collected with a Zeiss 510 confocal microscope. For quantitation of ATP7A intracellular localization (Fig. 8D), 550–600 cells from 17 to 19 independent confocal images per transfection were scored, and the sample means and standard deviations calculated.

Fluorescence-activated cell sorting

Fixed, non-permeabilized fibroblasts (typically 105 cells) were incubated for 1 h at 25 °C with a carboxyl-terminal ATP7A antibody in 0.1 ml of PBS containing 1% BSA. Flow cytometric data acquisition was carried out using a dual laser four-color Becton Dickinson FACSort flow cytometer. Data analysis was performed using FloJo v7.1 (Tree Star Inc., San Carlos, CA, USA) software. Triplicate experiments were performed.

Sucrose gradient experiments

Transfected Hek293T cells (2×105) were lysed on ice in 0.8 ml of lysis buffer [25 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l NaF and 1 mmol/l sodium orthovanadate and the protease inhibitor mix Complete Mini (Roche)], dounce homogenized 10 times on ice and mixed with 0.8 ml of 80% sucrose made with lysis buffer. After transfer to centrifuge tubes, lysates were overlaid with 2 ml of 30% sucrose in lysis buffer, followed by 1 ml of 5% sucrose in lysis buffer. After centrifugation for 16 h at 114 562 g in a Beckman SW50.1 rotor, 400 μl fractions were collected from the top of the gradient. Levels of ATP7A, transferrin receptor and flotillin 1 in each fraction were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting.

Statistical analyses

Data from siRNA and flow cytometry experiments were analyzed by two-tailed Student’s t-tests. P values < 0.05 were considered statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We are grateful to the patients and their families for their kind collaboration. We thank the NICHD Microscopy & Imaging Core for facility use, the NICHD Biomedical Mass
Spectrometry facility for peptide analysis, Peter Steinbach, Center for Molecular Modeling, Center for Information Technology, NIH for helpful discussions and the PDB file from which images in Figure 10 were generated, Tanya Lehkey, NINDS, for expert performance of nerve conduction studies and Tracey Rouault and Jerry Strauss for critical review of the manuscript. We dedicate this paper to the memory of our friend and colleague Jim Garbern, whose untimely death occurred while the manuscript was in review.

FUNDING

The work was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health.

REFERENCES


