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Copper-transporting ATPases ATP7A and ATP7B: cousins, not twins

Rachel Linz · Svetlana Lutsenko

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Abstract Copper plays an essential role in human physiology and is indispensable for normal growth and development. Enzymes that are involved in connective tissue formation, neurotransmitter biosynthesis, iron transport, and others essential physiological processes require copper as a cofactor to mediate their reactions. The biosynthetic incorporation of copper into these enzymes takes places within the secretory pathway and is critically dependent on the activity of copper-transporting ATPases ATP7A or ATP7B. In addition, ATP7A and ATP7B regulate intracellular copper concentration by removing excess copper from the cell. These two transporters belong to the family of P_1-type ATPases, share significant sequence similarity, utilize the same general mechanism for their function, and show partial colocalization in some cells. However, the distinct biochemical characteristics and dissimilar trafficking properties of ATP7A and ATP7B in cells, in which they are co-expressed, indicate that specific functions of these two copper-transporting ATPases are not identical. Immunodetection studies in cells and tissues have begun to suggest specific roles for ATP7A and ATP7B. These experiments also revealed technical challenges associated with quantitative detection of copper-transporting ATPases in tissues, as illustrated here by comparing the results of ATP7A and ATP7B immunodetection in mouse cerebellum.

Keywords ATP7A · ATP7B · Copper · Wilson disease · Menkes disease · Trafficking · Transport

Introduction

Cu-transporting ATPases (Cu-ATPases) ATP7A and ATP7B are essential for human copper metabolism. Numerous physiological processes in the human body such as respiration, detoxification of radicals, neurotransmitter biosynthesis, iron transport, and others rely on the activity of cuproenzymes. For those copper-dependent enzymes that are secreted or targeted to the plasma membrane, incorporation of cofactor (copper) takes place within the secretory pathway. The mechanism of biosynthetic incorporation of copper into these proteins is yet to be fully understood, however the critical role of copper-transporting ATPases in this process is certain (Mishima et al. 1999; Petris et al. 2000; El Meskini et al. 2003; Terada et al. 1998). ATP7A and ATP7B use the energy of ATP-hydrolysis to transfer copper from the cytosol into the lumen of the secretory pathway (Voskoboinik et al. 1999; Voskoboinik et al. 2001). This process is disrupted in human genetic disorders associated with inactivating mutations in ATP7A (Menkes disease) and ATP7B (Wilson's disease). Inactivation of Cu-ATPases results in the loss of activity of such enzymes as tyrosinase (Mishima et al. 1999; Petris et al. 2000), lysyl oxidase (Kuivaniemi et al. 1982; Royce and Steinmann 1990), peptidyl-α-monooxygenase (El Meskini et al. 2003; Prohaska et al. 1997) (ATP7A inactivation) and ferroxidase ceruloplasmin (Terada et al. 1998; Meng et al. 2004) (loss of ATP7B activity).

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R. Linz · S. Lutsenko
Department of Biochemistry and Molecular Biology,
Oregon Health & Science University,
Portland, OR 97239, USA

S. Lutsenko (✉)
Oregon Health & Science University,
Portland, OR 97239, USA
e-mail: lutsenko@ohsu.edu

In addition to their critical contribution to the biosynthesis of copper-dependent enzymes, ATP7A and ATP7B play an equally important role in regulating copper levels in the body and in maintaining intracellular copper concentration. Copper absorption critically depends on ATP7A. In intestinal cells, ATP7A exports copper from the enterocytes into circulation for further distribution throughout the body (Nyasae et al. 2007). Inactivation of ATP7A in Menkes disease results in increased copper concentration in the intestine (Horn and Jensen 1980; Kodama et al. 1999) due to diminished copper export and poor copper supply of other tissues, with brain and connective tissues being particularly affected (Kodama et al. 1999). Removal of excess copper from the body requires function of ATP7B in the liver (Fanni et al. 2005). ATP7B facilitates transport of copper from the hepatocytes into the bile (Malhi et al. 2002), and this process is required for homeostatic control of copper in the body (Schilsky et al. 2000). Inactivation of ATP7B in Wilson's disease is associated with massive copper overload in the liver and some other tissues, and copper-induced toxicity (Das and Ray 2006).

Analysis of tissue-specific expression has yielded first hints to specific functions of ATP7A and ATP7B in tissues The distinct roles for ATP7A and ATP7B in mammalian copper homeostasis were first proposed by Kuo and co-workers who analyzed the developmental expression of the corresponding genes during mouse embryonic development using RNA *in situ* hybridization (Kuo et al. 1997). These studies revealed ubiquitous expression of murine ATP7A throughout the embryo during gestation, leading the authors to the conclusion that ATP7A functions in the "individual homeostatic maintenance of copper levels in all cell types". The expression of ATP7B in the embryo showed clear tissue specificity with particularly high expression of ATP7B in the liver, strong staining in the villous epithelium of intestine, of the lining of the respiratory tract, especially bronchial epithelium (Kuo et al. 1997). Expression in the heart and thymus were also observed, while no staining of ATP7B in the brain or kidney was detected. Tissue-specific expression of ATP7B led to the suggestion that the role of this protein is likely to be specialized and may differ for different tissues. One of such roles could be the ATP7B mediated delivery of copper to ceruloplasmin, a copper-dependent ferroxidase. This conclusion is supported by a significant overlap in the patterns of expression for ATP7B and ceruloplasmin (Klomp et al. 1996; Jaeger et al. 1991).

The results of the insightful study by Kuo and colleagues raised an interesting question about individual roles of each Cu-ATPase in tissues, in which these two transporters are co-expressed. As a result of biochemical investigations, it has become clear that the enzymatic characteristics of ATP7A and ATP7B are not identical (Barnes et al. 2005). Both ATP7A and ATP7B are members of the P1-type ATPases family of transporters and use the energy of ATP hydrolysis to transport copper across cell membranes. The reaction involves the transfer of γ-phosphate to catalytic Asp residue, the transient formation of phosphorylated intermediate, and subsequent hydrolysis of this intermediate by water. The characterization of time-dependences for these individual steps revealed that the formation of phosphorylated intermediate and the dephosphorylation step are both faster for ATP7A compared to ATP7B (Barnes et al. 2005). This result suggested that ATP7A could have a higher turnover rate and could transport more copper per minute compared to ATP7B. This difference could be one of the reasons why ATP7A is a house-keeping enzyme, while ATP7B is more specialized.

Another key difference between ATP7A and ATP7B was revealed upon analysis of their intracellular behavior (for most recent review of Cu-ATPase intracellular targeting and trafficking, see La Fontaine and Mercer (La Fontaine and Mercer 2007)). It has become clear that in many cells, in which Cu-ATPases are co-expressed, under basal growth conditions both Cu-ATPases have intracellular perinuclear localization and show significant, but incomplete, overlap (Fig. 1). Studies using various protein markers indicate that the compartment(s), in which ATP7A and ATP7B are present, belong to the *trans*-Golgi network (TGN) (Nyasae et al. 2007; Guo et al. 2005). The increase in the intracellular copper concentration induces re-localization of Cu-ATPases. In polarized epithelial cells, such as intestinal Caco-2 cells or MDCK cells, ATP7A traffics from the TGN towards the basolateral membrane (Nyasae et al. 2007; Greenough et al. 2004), although most of the protein remains intracellular (Nyasae et al. 2007) unless it is overexpressed. In tissues, the copper-dependent relocalization also leads to accumulation of ATP7A in sub-basolateral vesicles (Monty et al. 2005) and this step is thought to be associated with copper export into the blood (Ke et al. 2006).

Similarly to ATP7A, the intracellular localization of ATP7B changes in response to copper elevation, however the trafficking pattern is different. In polarized hepatocytes, ATP7B traffics towards the apical membrane and accumulates in the sub-apical vesicles (Guo et al. 2005; Bartee and Lutsenko 2007; Cater et al. 2006; Schaefer et al. 1999). Although some overlap between ATP7B and apical membrane marker MRP2 can be detected (Guo et al. 2005; Bartee and Lutsenko 2007; Roelofsen et al. 2000), the majority of ATP7B remains intracellular (Gou et al. 2005; Bartee and Lutsenko 2007; Cater et al. 2006). This trafficking behavior led to the model in which the Cu-ATPase-mediated export of copper from the cells involves several steps: sensing elevated copper and relocalization to

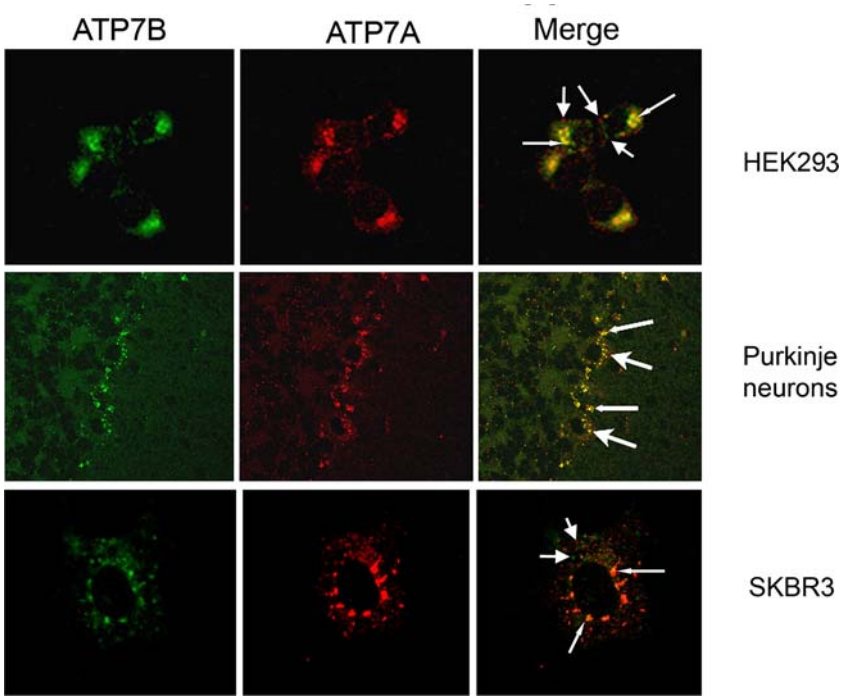

Fig. 1 ATP7A and ATP7B are co-expressed in various cells and show partially overlapping intracellular localization. Staining of ATP7A and ATP7B using specific antibodies is shown in cultured HEK293 (renal) cells, SKBR3 (mammary carcinoma) cells and in Purkinje neurons in tissue. The small-head arrows indicate co-localization of ATP7A and ATP7B; while big-head arrows point to distinct, non-overlapping localization

the vesicles, the sequestration of copper into the vesicles, and the fusion of vesicles with the plasma resulting in copper release. Which of these steps is rate limiting is not entirely clear. Similarly, it remains unknown whether upon exocytosis copper is released in a form of free ion or it is bound to some carrier molecule that was present within the vesicles. It has been shown that ATP7A and ATP7B return back to the TGN when excess copper is removed.

The trafficking of ATP7A and ATP7B to different membranes led to the suggestion that in cells co-expressing both Cu-ATPases, the role of these proteins is to export copper across the basolateral and apical membranes, respectively. This idea received some support in recent studies of cultured placental cells. In differentiated polarized Jeg-3 cells, an *in vitro* model of placental trophoblasts, both ATP7A and ATP7B are expressed (Hardman et al. 2007). Interestingly, treatment of Jeg-3 cells with insulin results in very different regulation of two Cu-ATPases. The ATP7A mRNA and protein levels are increased in response to treatment with hormone, and ATP7A protein is found relocalized towards the basolateral membrane. This trafficking event is associated with the increased transport of copper across the basolateral membrane (Hardman et al. 2001). By contrast, the levels of ATP7B are decreased in response to insulin, the protein is detected in perinuclear compartment, and the transport of copper across apical membrane is diminished (Hardman et al. 2001). These interesting results are consistent with the involvement of ATP7A in basolateral export of copper in response to treatment with insulin. The diminished copper transport across the apical membrane, however, can be due to lower amount of ATP7B or it may also reflect increased activity of ATP7A at the basolateral membrane and lower availability of copper for ATP7B. Whether ATP7B traffics to the apical membrane in Jeg-3 cells in response to copper elevation has not yet been reported.

It is also important to note that the trafficking of endogenous ATP7B in response to copper elevation or any other signals in cells other than hepatocytes has not been firmly established. Our studies in cultured and primary kidney cells in which both ATP7A and ATP7B are co-expressed show that renal ATP7B does not traffic at any copper concentrations in either polarized or non-polarized renal cells (Barnes et al, manuscript in preparation). It could be that in some cells, such as epithelial renal cells, in addition to (or instead of) copper export across the apical membrane, ATP7B fine-tunes intracellular copper by sequestering it into the intracellular vesicles.

Do copper-ATPases compensate for the lack of each other function? In vitro, the defects in ATP7A function can be compensated by ATP7B. This can be demonstrated by using fibroblasts isolated from Menkes disease patients, which accumulate copper unless either ATP7A or ATP7B are heterologously expressed (Lockhart et al. 2002). Similarly, in some tissues, for example cerebellum, the lack of ATP7B activity appears to be compensated by ATP7A (Barnes et al. 2005). The lack of ATP7A function, however, is not compensated by ATP7B (Niciu et al. 2007). In intestinal cells, the presence of ATP7B does not prevent

copper accumulation due to ATP7A inactivation. Partially, this could be due to the lower efficiency of ATP7B as a transporter or it could be that in intestinal cells (as in renal cells) ATP7B does not traffic towards the plasma membrane and is largely involved in vesicular sequestration. Better understanding of the ATP7B trafficking in various cells lines as well as the role of ATP7B in copper export in cells/tissues, in which apical copper export is insignificant, would be very important.

Challenges in quantitative determination of expression and localization of ATP7A and ATP7B in tissues Immunocytochemistry and immunohistochemistry experiments have yielded a wealth of useful information on the localization and targeting of Cu-ATPases in various cells (La Fortaine and Mercer 2007). However, deriving quantitative conclusions from the analysis of ATP7A and ATP7B in tissues has been challenging. This can be illustrated by the results of the experiments on localization of ATP7A in mouse cerebellum. In adult cerebellum, DIG-labeled RNA probes detected ATP7A mRNA in the granular layer of the cerebellum and in Purkinje neurons (Murata et al. 1997). The biotin-labeled RNA probes and antibody showed staining of ATP7A mRNA and protein in glial cells (Barnes et al. 2005), with very little, if any, staining of Purkinje neurons (PN). Diaminobenzidine-based visualization of immunostaining showed high expression of ATP7A in PN with low levels in cells of the granular layer (Niciu et al. 2006). Clearly, a single method of localization/visualization may not provide the most accurate information on the expression pattern.

The comparison of two studies utilizing the same anti-ATP7A antibodies is particularly instructive (Barnes et al. 2005; Niciu et al. 2006). Expression of ATP7A in the brain appears to be highest in the early postnatal period (P2 – P4) (Niciu et al. 2006). At this age, ATP7A was detected in PN by either diaminobenzidine-based color detection (Niciu et al. 2006) or by immunofluorescence (Barnes et al. 2005). Subsequent changes in the expression of ATP7A during development were also observed in both studies, however, fluorescence-based immunohistochemistry and *in situ* hybridization showed the decrease in a expression of ATP7A mRNA and protein in PN with a concomitant increase of fluorescent signal in adjacent Bergmann glia cells (Barnes et al. 2005). In contrast, the diaminobenzidine-based immunodetection revealed a significant increase in PN and no glial staining (Niciu et al. 2006). Since the same anti-ATP7A antibody were used in both studies, the variance in levels of ATP7A in adult PN can only be linked to differences in fixing and detection protocols.

That aldehyde fixation time has a great and different effect on immunohistochemical detection of ATP7A and ATP7B in PN is illustrated in Fig. 2 (left panels). The anti-ATP7A antibody shows significantly more intense staining of PN cell bodies after four hours of fixing compared to 24 hour fixing time, consistent with both previously published results (Barnes et al. 2005; Niciu et al. 2006). Yet, in younger animals (P2 or P18) the 24-hours fixing protocol yields clear staining of PN (Barnes et al. 2005). Thus, the age-dependent changes in tissue may significantly alter the efficiency of detection. Interestingly, regardless of fixing time, in adult cerebellum the anti-ATP7A antibody shows a

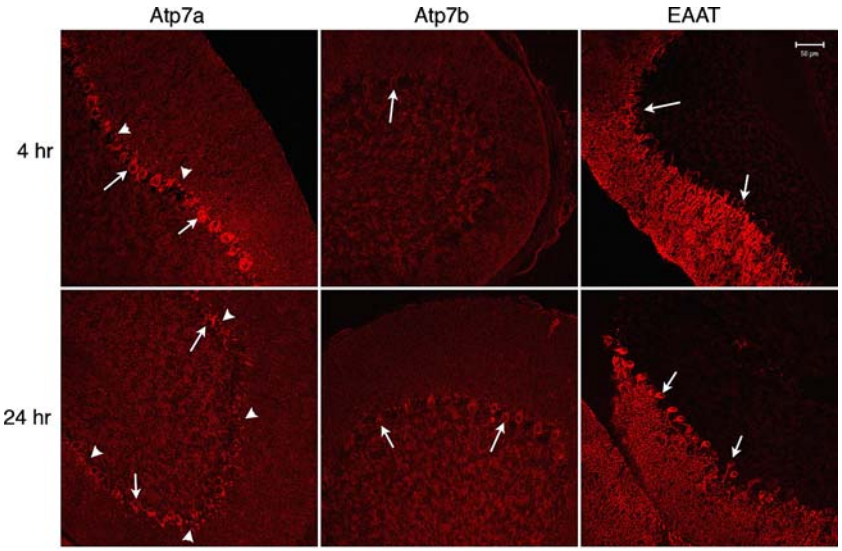

Fig. 2 The effect of the fixation on the intensity and the pattern of staining for ATP7A, ATP7B and EAAT4 in mouse Purkinje neurons. Cerebellum was fixed with paraformaldehyde for either 4 hours (as in (Niciu et al. 2006)) or for 24 hours (as in (Barnes et al. 2005)); the tissue was then sliced and stained with anti-ATP7A, anti-ATP7B, or anti-mEAAT4 (glutamate transporter and a known PN resident protein) under identical conditions. Visualization – using fluorescently labeled secondary antibody. The arrows indicate the cell bodies of Purkinje neurons, the triangles point to cell bodies of neighboring glial cells. The intensity of ATP7A in Purkinje neurons vary from weak (shown) to non-detectable (Barnes et al. 2005)

strong signal in the Bergmann glia when paired with a fluorescently labeled secondary antibody. Pre-incubation of anti-ATP7A antibody with the antigen only partially diminishes the glial staining, while the decrease in the staining of PN is more pronounced (our data, not shown). Whether this difference is due to nonspecific staining of Bergmann glia or increased sensitivity of the fluorescent detection is still unclear.

The quantitative comparison of ATP7A and ATP7B expression in the same tissue can also be challenging, as illustrated in Fig. 2. The time-dependence of staining for ATP7B (Fig. 2 middle panels) and for a well-known resident of PN, glutamate transporter EAAT (Fig. 2 right panels), is opposite to that of ATP7A (Fig. 2, left panels). Both anti-ATP7B and anti-mEEAT4 show more intense staining of PN after 24 hours of fixing and very little PN cell body staining after four hours (Fig. 2). Therefore, in this particular case, the co-staining of ATP7A and ATP7B in the same tissue using the same fixing time only yields distribution patterns for each transporter but not their relative levels of expression.

In summary, ATP7A and ATP7B are essential copper transporters with distinct distribution in tissues. Their localization in the TGN under basal conditions is consistent with the role in biosynthetic delivery of copper to the secretory pathway. The partial overlap in the intracellular localization of ATP7A and ATP7B is likely to reflect their different sorting and trafficking fates. The quantitative analysis of the levels of Cu-ATPases in tissues remains to be optimized. Nevertheless, the available immuno-detection data consistently point to the key role of ATP7A in the basolateral export of copper (Nyasae et al. 2007; Monty et al. 2005; Ke et al. 2006) and the importance of ATP7A function at the early stages of development, particularly in the brain (Niciu et al. 2006; Niciu et al. 2007; El Meskini et al. 2005). The specific role of ATP7B in tissues, in which it is co-expressed with ATP7A, may involve the delivery of copper to ceruloplasmin, apical copper export and/or fine-tuning of intracellular copper by sequestration of copper in the vesicles.

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