Function and Regulation of Human Copper-Transporting ATPases

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I. INTRODUCTION: THE ESSENTIAL ROLE OF COPPER-TRANSPORTING ATPases ATP7A AND ATP7B IN HUMAN PHYSIOLOGY

Copper-transporting ATPases (Cu-ATPases) are essential for human growth and development. Numerous physiological processes depend on adequate and timely transport of copper mediated by these proteins. Cu-ATPases are polytopic membrane proteins that translocate copper from the cytosol across cellular membranes using the energy of ATP hydrolysis. This transport process serves to reduce the intracellular copper concentration and also to contribute to homeostatic control of copper in the body. In addition, Cu-ATPases perform important biosynthetic functions by delivering copper into the secretory pathway where the metal ion is incorporated into copper-dependent enzymes, such as dopamine-β-hydroxylase, tyrosinase, lysyl oxidase, peptidylglycine-α-amidating monoxygenase, ceruloplasmin, and others.

The physiological importance of Cu-ATPases in humans can be illustrated by the deleterious consequences of the Cu-ATPase inactivation on cell metabolism. Mutations or deletions in the gene encoding the Cu-ATPase ATP7A are associated with a fatal childhood disorder, Menkes disease (OMIM 309400). Patients with Menkes disease display dramatic developmental and neurological impairment due to disrupted delivery of copper to the brain (114). In addition, the patients have a variety of other symptoms that are caused by decreased function of copper-dependent enzymes and include connective tissue abnormalities, lack of pigmentation, and tortuosity of blood vessels (114, 130, 131, 266, 281, 297). The majority of Menkes disease patients die in early childhood.

Mutations in the gene encoding the Cu-ATPase ATP7B also result in a severe metabolic disorder, known as Wilson disease (OMIM 277900). The phenotypic manifestations of this disorder differ from those of Menkes disease. ATP7B inactivation is associated with copper accumulation in several tissues, particularly in the liver and the brain, and a spectrum of hepatic and neurological abnormalities. These may include liver dysfunction or failure, movement disorders, and psychiatric manifestations (65, 75, 90). Although copper chelation therapy using d-penicillamine is available for treatment of Wilson disease, this therapy is not always successful, especially if diagnosis is delayed, and may have severe side effects (28, 110, 201). In recent years, other therapies have been developed, including treatments with trientine, tetrathiomolobdate, or zinc; however, their efficacy and potential side effects remain to be fully evaluated (29, 182, 259).

The two diseases of copper metabolism illustrate the fundamental need for tight homeostatic control of intracellular copper as either copper deficiency (Menkes disease) or copper accumulation (Wilson disease) are extremely deleterious to cell function. Such precise control is mediated through the coordinated action of several proteins, including the high-affinity copper uptake protein Ctr1, a set of small cytosolic copper carriers called metallochaperones, which distribute copper to various cell destinations, and Cu-ATPases. Descriptions of cellular machinery involved in copper uptake and in the subsequent distribution of copper can be found in a number of excellent recent reviews (96, 178, 209, 220). In this review we focus primarily on the physiological role of Cu-ATPases in various tissues and the molecular mechanisms of their function and regulation. Clinical aspects of Menkes disease and Wilson disease as well as currently available animal models have been described in recent literature (66, 90, 130, 131, 175, 250, 280, 281) and are not discussed here.

II. COPPER DISTRIBUTION IN TISSUES

A. Major Pathways of Copper Distribution

1. Copper uptake in intestine

Copper plays a critical role in human metabolism as a cofactor of key metabolic enzymes, which are involved in respiration, neurotransmitter biosynthesis, radical detoxification, iron metabolism, and many other physiological processes. The average daily intake of copper is between 1 and 3 mg, and this amount is adequate for body needs. The majority of copper absorption appears to take place in the duodenum; however, the molecular pathways through which dietary copper is absorbed by the intestinal epithelium are not well understood. The high-affinity copper transporter Ctr1 has been detected at the apical membrane of intestinal cells in agreement with a role in copper uptake from the lumen (139). However, this localization is seen only in suckling mice, while in adult animals Ctr1 has mostly an intracellular localization (139). This observation suggests a role for Ctr1 in regulated, rather than constitutive, copper transport and/or possible involvement of Ctr1 in the export of copper from intracellular stores for further utilization by the cell.

These possible roles of Ctr1 in the intestinal copper uptake were highlighted in the experiments on targeted deletion of Ctr1 in mouse intestine. Genetic inactivation of Ctr1 was shown to effectively block copper absorption.
into the blood and result in copper deficiency in other tissues (195). However, the deletion did not prevent copper entering and accumulating in the intestinal cells (195). This observation rules out the essential role for Ctrl in apical uptake of copper, indicating that other pathways/transporters could be involved.

The presence of low-affinity copper transporter in Ctrl−/− embryonic cells has been demonstrated (149). One of the candidates for such a transporter is a relatively nonselective, divalent metal transporter DMT1 (12, 67), which mediates intestinal uptake of dietary iron (8). The siRNA-mediated knock-down of DMT1 in cultured cells significantly decreases both iron and copper uptake (12); whether DMT1 transports copper in tissue has not been directly examined. Another candidate is the ATP-driven copper transporter system detected at the brush-border membrane (127); however, the molecular nature of this putative Cu-ATPase remains unknown. Lastly, such mechanisms of copper uptake as pinocytosis may also play a role in copper uptake (172), particularly during maturation of the gastrointestinal tract.

Copper is exported from the enterocytes into the blood by Cu-ATPase ATP7A (Fig. 1) in a process that involves trafficking of the transporter towards the basolateral membrane (184, 235). In Menkes disease, ATP7A is inactivated and copper export from the enterocytes is greatly impaired. As a result, copper accumulates in intestinal cells and less copper is delivered to the blood, resulting in restricted copper supply to other tissues (131). A close relationship appears to exist between iron and copper absorption in the intestine (160, 316). Under conditions of iron deficiency in rats, DMT1 expression is increased and could be responsible for the increase in absorption of both iron and copper at the brush border (44). The expression of ATP7A in duodenum is also markedly elevated in iron deficiency, most likely contributing to the overall increase of copper transport across intestinal epithelium (44, 235).

Current structural data as well as dependence of copper trafficking proteins on reducing reagents suggest that copper enters the cell, migrates within the cell, and then is exported from the cell in the reduced Cu(I) form (148, 158, 231, 234, 290, 305). However, in both the intestinal lumen and in the serum, copper is thought to be present in the oxidized Cu(II) form, and therefore, copper has to be reduced upon entry into the cell and oxidized when exiting the cell. The existence of a cytochrome b protein with copper reductase activity has been demonstrated in rabbit enterocytes (128). Such reductase activity could be an important component of the copper uptake machinery. It is not yet clear whether oxidase activity is required for copper exit from the cell and whether the transport of copper by ATP7A and ATP7B is coupled to such copper oxidase activity.

2. ATP7B in hepatocytes is essential for homeostatic regulation of copper in the body

The majority of copper that emerges from the intestinal epithelium into the blood is delivered to the liver...
(23), and less to kidney and other tissues (159). The liver is the central organ of copper homeostasis and is primarily responsible for the export of excess copper out of the body (Fig. 1). The copper uptake into the liver does not appear to be highly regulated. In contrast, the export of copper from the liver is a regulated copper-dependent process, which is mediated by a copper-transporting ATPase ATP7B.

After entry into hepatocytes, copper is distributed to various intracellular destinations (Fig. 2). In the cytosol, copper is utilized by a radical-detoxifying enzyme copper, zinc-dependent superoxide dismutase (SOD1), which acquires copper with the help of a specific metallochaperone, copper chaperone of superoxide dismutase (CCS) (45). Copper also enters the mitochondria, where it is incorporated into cytochrome-c oxidase (COX). Several candidate proteins have been proposed to contribute to this latter process (101, 221, 223); however, the exact mechanism of copper delivery to the mitochondria is not yet understood.

The third important destination of copper in a cell is the secretory pathway. Distinct compartments of the secretory pathway, the trans-Golgi network (TGN), contain Cu-ATPases (ATP7B in hepatocytes), which receive copper from the cytosolic copper chaperone Atox1 (86, 87, 300). The ATPases then transfer copper across the membrane into the lumen of the TGN (Fig. 2). In hepatocytes, following transport into the lumen of the secretory pathway, copper is incorporated into the copper-dependent ferroxidase ceruloplasmin (CP), which is subsequently secreted into the blood (Fig. 1). CP is the major copper-containing protein in a serum; however, it does not seem to play an essential role in copper metabolism (180). The CP−/− knockout mice do not show marked abnormalities in copper homeostasis; however, they do accumulate copper in the liver (180). In addition, 67Cu-labeled ceruloplasmin was shown to be more of an effective donor of copper compared with ionic metal (151). Therefore, the regulatory involvement of CP in copper metabolism cannot be excluded (see more on CP below).

Excess cytosolic copper is excreted into the bile in a form that is less easily reabsorbed (159). In rats, biliary excretion can be detected as early as 15 min after gastrointestinal injection of the Cu isotope, although more time is necessary to reach saturation (23). ATP7B is required for copper excretion into the bile, and to perform this function, ATP7B relocalizes towards canalicular membrane of hepatocyte (for details on intracellular trafficking of Cu-ATPases, see sect. vi). ATP7B-dependent copper export into the bile and subsequently to feces represents the major route of copper excretion from the body. In Wilson disease patients, both copper transport to the secretory pathway and copper release into the bile are greatly impaired (Fig. 1), resulting in marked accumulation of copper in the liver, very low levels of copper-bound ceruloplasmin in the serum, and low biliary copper (146, 252, 267).

3. Copper distribution in nonhepatic tissues

Experiments in rats using radioactive copper revealed that newly absorbed copper appears in the bloodstream in two waves: an initial peak (after 2 h) corresponds to copper exiting the intestine, and the second peak (after ~6 h) represents copper incorporated into ceruloplasmin, which is secreted by the liver (159). All serum copper is thought to be bound to protein carriers (161) and, possibly, low-molecular-weight compounds. In vitro, copper can be presented to a cell in various forms (as a free ion, or in a protein-, peptide-, or amino acid-bound form), and in all these cases, copper uptake takes place. In which form copper is recognized by the uptake machinery in vivo is yet to be fully understood.

Copper, when delivered to various organs, is utilized to produce copper-dependent enzymes with general (cytochrome-c oxidase or superoxide dismutase) and tissuespecific functions [for example, dopamine-β-hydroxylase in the adrenals (74), peptidylglycine α-amidating monoxygenase in the pituitary (262), or tyrosinase in melanocytes (213)]. The set of proteins regulating copper distribution within the cells is thought to be the same in all tissues (Fig. 2). Some organs, though, express only one Cu-ATPase [for example, ATP7A in adrenal gland (74) or ATP7B in hepatocytes (270)], and in these tissues, a

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**FIG. 2.** Intracellular pathways of copper distribution. Copper enters the cell through the high-affinity copper transporter Ctr1 and binds to cytosolic copper chaperones. Cox17 may participate in delivery of copper to the mitochondrion, although this role has recently been questioned (174), and together with Sco proteins facilitates incorporation of copper into cytochrome-c oxidase (COX). CCS transfers copper to cytosolic SOD1. The red arrows indicate the pathway in which Cu-ATPases play the major role. In this pathway, Cu-ATPases receive copper from ATOX1, transfer copper into the lumen of the secretory pathway, and also export excess copper from the cell.
single Cu-ATPase appears to perform both biosynthetic and copper export functions.

Many tissues, however, such as brain, developing kidney, placenta, mammary gland, eye, lung, and some others, express both Cu-ATPases [for details on expression profiles, see NCBI database UniGene Hs. 496414 (ATP7A) and UniGene Hs. 492280 (ATP7B)]. The cell specificity of Cu-ATPase expression as well as the presence of two Cu-ATPases in the same cells can be associated with different functional characteristics of ATP7A and ATP7B, their distinct developmental regulation, or different targeting and trafficking behavior in polarized epithelia. Recent studies (see sect. iii) suggest that these differences between ATP7A and ATP7B could be linked to their distinct physiological roles in tissues. The conclusion that the roles of two human Cu-ATPases are not identical is most apparent when one considers intestine. In the intestine, both Cu-ATPases are expressed (20, 138, 165), and ATP7B does not compensate for the lack of ATP7A function, as evident from the Menkes disease phenotype. In contrast, in the cerebellum of Atp7b−/− mice, ATP7A appears to substitute for missing ATP7B (19), and in vitro, the ATP7A inactivity can be suppressed by heterologous expression of ATP7B (144, 164). Determining specific roles for each ATPase and the extent of functional complementation in vivo is important as it may help to better understand consequences of ATP7A and ATP7B inactivation in such tissues as lung, heart, and kidney.

B. Function of Cu-ATPases in Copper Delivery to the Secretory Pathway

The first evidence for ATPase-mediated copper transport to the secretory pathway was obtained in the yeast Saccharomyces cerevisiae that contains the ATP7A/ATP7B homolog Ccc2p (315). Ccc2p is located in the late Golgi compartment, where it transports copper from the cytosol to the Golgi lumen for biosynthetic incorporation into Fet3p (a copper-dependent plasma membrane metallo-oxidase involved in iron uptake). Genetic deletion of ccc2 abolishes copper incorporation into Fet3p and perturbs iron uptake, as illustrated by the inability of Δccc2 cells to grow under iron-limiting conditions (315). Heterologous expression of either ATP7A or ATP7B restores the copper-dependent Fet3 activity and permits the growth of ccc2 mutants on an iron-deficient medium (61, 102, 104, 107). Complementation of the Δccc2 phenotype serves as a convenient screening assay for evaluation of the ATP7A and ATP7B activity (for details, see sect. viA).

The initial results showing ATP7A/ATP7B-mediated transport of copper to the secretory pathway in yeast were further confirmed by more recent studies in mammalian cells, which yielded direct evidence for the role of Cu-ATPases in the biosynthesis of copper-dependent enzymes (see below). The biosynthetic events utilizing the Cu-ATPase function were shown to take place in specific compartments of Golgi, require prefolded acceptor protein, and be regulated by copper (26, 61, 134, 216, 248, 260, 262). The mechanism of coupling between copper transport and copper incorporation into target proteins is not understood, although it does not seem to involve additional proteins (61). In some cases, the Cu-ATPase and an acceptor protein can be coimmunoprecipitated, as was shown for ATP7A and superoxide dismutase 3 (SOD3) (230). This observation points to close spatial proximity of the Cu-ATPase and the copper-accepting enzymes (230) and also raises an interesting possibility that copper binding to the acceptor protein modulates its protein-protein interactions with the transporter. Much remains to be learned about the mechanism of metallation of copper-dependent enzymes and coordination of their biosynthetic rates with the transport activity of Cu-ATPases. Here we will briefly describe the copper-dependent enzymes for which dependence on Cu-ATPase function has been directly demonstrated and discuss how this information is being used for better understanding of the Cu-ATPase function.

1. Copper transfer to tyrosinase can be used to measure Cu-ATPase activity in cells

An elegant and convincing demonstration of the direct role of Cu-ATPases in the delivery of copper to the secretory pathway of mammalian cells was provided by Petris et al. (213) using tyrosinase as an example. Tyrosinase is a copper-dependent enzyme that participates in the formation of pigment melanin by catalyzing the hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA-quinone. The incorporation of copper into two binding sites takes place during tyrosinase passage through the secretory pathway and is required for its activity. Menkes disease patients are known to have abnormal pigmentation, which can be partially corrected by subcutaneous copper injections (258). Therefore, it was proposed that ATP7A, which is not functional in Menkes disease patients, was responsible for delivering copper to tyrosinase (213).

This prediction was experimentally verified using fibroblasts derived from the skin of Menkes patients (Menkes fibroblasts, Me32, Me52). The heterologous expression of tyrosinase in these fibroblasts yields an inactive apo-tyrosinase; however, the biosynthesis of copper-bound tyrosinase and the generation of the pigment are restored by the transfection of the ATP7A-expression construct into these cells (213). Altogether, these functional data and the Menkes disease phenotype indicate that one of the physiological functions of ATP7A is to metallate apo-tyrosinase. ATP7B does not seem to play a
role in copper delivery to tyrosinase in tissues; however, in an in vitro system, such as cultured hepatocytes, ATP7B can mediate copper delivery to heterologously expressed tyrosinase (85). Therefore, the measurements of tyrosinase activity can be used as a coupled assay to assess the ability of various ATP7A and ATP7B mutants to transport copper into the secretory pathway in mammalian cells.

2. Lysyl oxidase dependent processes are particularly sensitive to ATP7A inactivation

Copper-containing amine oxidases contain peptidyl-2,4,5-tri(oxo)phenylalanine (TOPA) at their active centers. TOPA is formed by copper-catalyzed oxidation of tyrosine, which takes place in Golgi or trans-Golgi (242). Mature lysyl oxidase catalyzes oxidative deamination of lysine and hydroxy-lysine residues in collagen and elastin; this step is required for subsequent protein cross-linking and polymer formation. Collagen and elastin fibers are important components of connective tissue and perform essential mechanical functions. The lack of functional ATP7A is associated with severe connective tissue defects in Menkes disease patients, likely due to disrupted delivery of copper to lysyl oxidase in the secretory pathway. The connective tissue defects including vascular tortuosity, loose skin, hyperextensible joints, and bone fragility (80, 136, 258) are commonly observed in Menkes disease patients.

The functional relationship between ATP7A and lysyl oxidase is further supported by the observation of a similar temporal expression of these two proteins during embryonic development in rats (272). It is notable that lysyl oxidase or lysyl oxidase-dependent processes are particularly sensitive to ATP7A inactivation. This conclusion was made based on studies of the occipital horn syndrome (OHS), a mild form of Menkes disease that primarily affects connective tissues (OMIM 304150). In one such study, Dagenais et al. (46) characterized a frameshift mutation of ATP7A in the OHS patient resulting in a COOH-terminal truncation of the protein. This mutation greatly diminished levels of ATP7A protein, but did not affect functional domains of the transporter. Unlike classical Menkes disease patients who show marked neurological impairment and die in early childhood, the OHS patient had relatively normal development and low-to-average cognitive abilities (46), suggesting that low level of ATP7A activity is sufficient to prevent severe neurological problems. At the same time, production of functional lysyl oxidase in the patient was diminished, and the patient displayed significant skeletal abnormalities including occipital horns, broad scapular necks, and radial bowing of the forearms (46). Similar phenotype has been observed in other OHS patients (84, 222, 225).

Studies in cultured cells offer a possible explanation for high sensitivity of connective tissue to ATP7A inactivation. It was shown that defect in ATP7A is associated not only with lysyl oxidase inactivation but also with dysregulation of mRNA transcription and/or turnover for such matrix proteins as elastin (72). Understanding the multifaceted role of ATP7A in connective tissues may have practical consequences. For example, precise control of intracellular copper levels has potential uses in tissue engineering to increase mechanical strength (47).

3. ATP7A-mediated copper delivery to peptidylglycine α-amidating monooxygenase does not require additional protein chaperones

Peptidylglycine α-amidating monooxygenase (PAM) is a copper-dependent enzyme primarily expressed in the pituitary gland, adrenal medulla, atrium of the heart, and the central nervous system. The enzyme has an important physiological role in catalyzing the conversion of over half of all neuropeptides into α-amidated peptides (262). RT-PCR experiments demonstrate that tissues abundant in PAM, such as pituitary and adrenal gland, also express ATP7A (74, 262). ATP7A and PAM are both present in the TGN, suggesting that ATP7A provides copper to PAM in this compartment (262). Inactivation of ATP7A has important consequences for PAM function, as illustrated by experiments in the mottled-brindled mutant mouse that lack functional ATP7A (262). In these mice, protein levels of PAM are normal; however, the level of amidated peptides is markedly reduced consistent with diminished PAM activity due to the lack of copper cofactor. It was proposed that a deficiency in peptide amidation is an important contributing factor to the developmental problems associated with Menkes disease (262).

Studies of PAM have also examined whether copper transfer from the Cu-ATPase to the acceptor protein in the secretory pathway requires the presence of additional helper molecules or chaperones. It was shown that expressing the catalytic core of PAM (PHM) in yeast produced active holoenzyme. This process was dependent on the presence in cells of an active Cu-ATPase, Ccc2p, a functional homolog of ATP7A. Since yeast cells do not have PAM homologs, yet Ccc2p can supply copper to PHM, it was concluded that luminal Golgi chaperones may not be required for coupling copper release from the transporter and incorporation into the acceptor enzyme (61).

4. Ceruloplasmin biosynthesis and ATP7B function are interdependent

Ceruloplasmin (CP) is a multi-copper oxidase that couples the reduction of O₂ to H₂O with the oxidation of Fe(II) to Fe(III). Unlike tyrosinase or PAM, which receive their metal cofactor from ATP7A, CP acquires copper......
from ATP7B. Similarly to biosynthesis of other copper-dependent enzymes, copper incorporation into apo-CP takes place in the secretory pathway (273). The direct involvement of ATP7B in this process was confirmed in the experiments in which recombinant adenovirus was used to introduce ATP7B cDNA into LEC rats. These animals lack functional ATP7B and only produce the apo-form of CP. Infusion of the recombinant adenovirus restores copper incorporation into CP in the LEC livers, providing strong evidence for the role of ATP7B in ceruloplasmin biosynthesis (273).

Studies of CP biosynthesis also demonstrated that ceruloplasmin enters the late secretory pathway and incorporates copper in an “all-or-none” process (99). It was suggested that such cooperativity permits a more sensitive response to copper concentrations if copper supplies become limiting (99). Whether or not ATP7B directly interacts with CP and “recognizes” the properly folded intermediate is unknown. Precise fit of the transporter and acceptor protein is not essential for copper delivery, since mammalian Cu-ATPases can also deliver copper to the yeast CP homolog Fet3, although with a noticeably lower efficiency (102, 104, 204, 205).

It is interesting that genetic inactivation of CP has a significant effect on the maintenance of intracellular copper levels in hepatocytes, a process that is thought to be mediated by ATP7B. In CP−/− mice, hepatic copper content is increased while copper absorption and biliary copper excretion appear unaltered (179). These data suggest that in the absence of CP, the transport activity of ATP7B is insufficient to efficiently remove all copper from hepatocytes. This could be due to lower transport activity of ATP7B in the absence of CP. CP, when present, may facilitate copper transport by binding and/or oxidizing released copper and thus serving as a copper “sink.” Alternatively, and perhaps more likely, trafficking of ATP7B towards apical membrane may represent a rate-limiting step in the biliary copper export. In this case, in the absence of CP, significantly more copper would need to be removed via this pathway. The copper will be sequestered by ATP7B into vesicles, but will remain mostly in cells due to slow vesicle-mediated exocytosis. This would result in copper accumulation in hepatocytes even in the presence of fully functional ATP7B. (For details on Cu-ATPase trafficking, see section VI.)

III. TISSUE-SPECIFIC FUNCTIONS OF Cu-ATPases

A. Expression Patterns of Cu-ATPases in Tissues Correlate With Phenotypic Manifestations of Menkes Disease and Wilson Disease

Characterizing patterns of ATP7A and ATP7B expression is the first step towards dissecting their specific roles in tissues. The symptoms of Menkes disease and Wilson disease suggest some tissue-specific functions for the ATP7A and ATP7B, respectively. Expression of ATP7A in intestine and in choroid plexus (138, 184, 193, 194, 235) correlates well with the key role of ATP7A in the generalized delivery of dietary copper via intestine to the body and subsequently into the brain. Consistent with this role, Menkes disease patients present with severe neurological deterioration, seizures, and mental retardation. ATP7A is also abundantly expressed in vascular smooth muscle cells, vascular endothelial cells, and aorta (230) as well as in cerebrovascular endothelial (CVE) cells (228). Inactivation of ATP7A is associated with vascular abnormalities (78) and may drastically affect the integrity of pulmonary arterial system (80). The marked effect of ATP7A inactivation on vasculature is not limited to insufficient supply of copper to lysyl oxidase. Recent studies in brindled mice (a mouse model of Menkes disease) also revealed a 50% decrease in the activity of SOD3, a copper-dependent enzyme which modulates levels of extracellular superoxide ions in vasculature. It was hypothesized that ATP7A may contribute to regulation of superoxide in the vasculature by supplying copper to SOD3 (230).

Similarly to Menkes disease, the major phenotypic manifestations of Wilson disease, such as liver disease and neurological abnormalities, parallel the primary expression of the corresponding Cu-ATPase, ATP7B, in the liver and the brain. Hepatic ATP7B is involved in the biosynthetic and export of excess copper into bile. In the brain, the major functions of ATP7B are likely to be in the basal ganglia, midbrain, andpons since these regions are often affected in Wilson disease patients (243). Whether the function of ATP7B in these regions is biosynthetic and/or homeostatic is currently unknown.

Kayser Fleischer corneal pigment ring is a characteristic and diagnostic sign of Wilson disease observed in the vast majority of patients with neurological abnormalities. The appearance of this ring is thought to reflect deposition of copper. The copper deposits are likely caused by inactivation of ATP7B, which is expressed in the retinal pigment epithelium and in the ciliary body during retinal development (25, 135). It was proposed that in the retina ATP7B is required for production of holo-ceruloplasmin (135); whether this is the only role of ATP7B in the eye remains to be determined.

Northern blot analyses demonstrated coexpression of two Cu-ATPases in the brain and several other tissues, such as kidneys, lung, placenta, and mammary gland (31, 39, 177, 207, 215, 270, 297, 309), raising questions about specific role of these transporters in these tissues. The sections below describe current data on division of labor between ATP7A and ATP7B in cells and tissues where these two transporters are coexpressed.
B. Both ATP7A and ATP7B Are Involved in Copper Transport to Milk

The respective roles of ATP7A and ATP7B in mammary gland are better understood compared with other tissues and illustrate distinct but complementary functions of Cu-ATPases. Copper is essential for neonatal growth and therefore has to be exported into the milk. The importance of copper as a nutrient is evident from a 20-fold increase in the copper uptake into mammary tissue that occurs upon lactation (56). After entering the mammary gland, copper is rapidly transferred to milk. The entire process is initiated by copper being taken up from maternal circulation into mammary epithelial cells most likely by a high-affinity copper transporter Ctr1 (121). When in cells, copper is utilized to produce ceruloplasmin, which is synthesized in substantial amounts in the mammary gland (111) and then secreted into milk. Copper also appears to be excreted directly from epithelial cells into the lumen of alveoli (56).

The role of ATP7A in copper delivery to milk was addressed by localizing the transporter in control and lactating mammary gland. In luminal cells of nonlactating tissue, ATP7A is confined to a perinuclear compartment (3) consistent with the low need for copper export. In a lactating gland, ATP7A appears in a diffuse pattern in cells of the areola and ducts, suggesting trafficking of ATP7A towards the plasma membrane and involvement of ATP7A in the cellular efflux of copper into the milk (3). The trafficking of ATP7A from the intracellular compartment towards the plasma membrane was also demonstrated in vitro following prolactin treatment of HC11 cells (121). In rats, ATP7A was detected in both the luminal (apical) and serosal (basolateral) membranes (120) and was suggested to have a dual role: exporting copper into the milk and back to the maternal circulation.

The involvement of ATP7B in copper export from the mammary gland is evident from the phenotypes of toxic (tx) milk and Atp7b−/− mice. The tx mice have an inactivating mutation in ATP7B (143), and the tx dams produce milk with low copper content. The tx pups have reduced copper levels in the stomach and often die unless nursed by nonmutant mice (hence the term “toxic milk”) (181). Similar copper perinatal deficiency is observed in Atp7b−/− knock-out mice (30). Characterization of copper distribution in control animals demonstrated that the copper content in milk is three- to fourfold higher than in mammary gland (30). In contrast, in Atp7b−/− knock-out mice lacking functional ATP7B, copper accumulates in the mammary gland, and the copper content of milk is only 30% of the norm. Interestingly, some copper is being delivered into milk even in the absence of functional ATP7B (most likely by ATP7A); however, ATP7A does not fully compensate for the lack of ATP7B function.

It was proposed that the role of ATP7A at the plasma membrane is to directly export copper into the milk (120), while ATP7B in the mammary gland could be necessary to export copper in a CP-bound form. Recent studies investigating milk copper composition during early and late periods of lactation have led to an interesting alternative model, in which ATP7B was suggested to play a constitutive role in milk copper secretion by delivering copper to ceruloplasmin and to secretory vesicles, whereas ATP7A serves to facilitate copper export in response to hormonal stimulation (121).

This model is consistent with changes in trafficking behavior of both Cu-ATPases in mammary cells. Similarly to ATP7A, during lactation the intracellular distribution of ATP7B changes and can be seen as a granular diffuse cytoplasmic pattern in contrast to the perinuclear staining in nonlactating animals (181). Regulated trafficking of the Cu-ATPases has been described in cultured cells and tissues in response to changes in copper concentration or, more recently, to Ca2+ signaling and hormonal stimulation (see sect. vi for details). It is currently unknown whether the relocalization of ATP7A and ATP7B in mammary gland is triggered by increased intracellular copper levels upon lactation, and/or is caused by induction of signaling pathways activated by hormones produced during lactation. A kinase-mediated phosphorylation of ATP7B that correlated with the intracellular localization of the transporter has been previously demonstrated in hepatic cells (284). Whether or not the regulatory phosphorylation contributes to the localization and function of Cu-ATPases in mammary gland remains to be examined.

C. Cu-ATPases Are Likely to Contribute to Tight Homeostatic Control of Copper in Kidneys

The kidneys have one of the highest copper concentrations among organs (7–12 mg/g; Ref. 159) and show tight homeostatic control of their copper content. Compared with other tissues, the renal copper content is less affected by systemic copper deficiency caused by limited absorption via the intestine (195). Similarly, dietary copper overload does not significantly alter copper concentrations in kidneys (5), pointing to efficient mechanisms that regulate copper levels in this tissue. It is thought that under normal conditions little copper is filtered in the glomerulus, since most serum copper is bound to proteins with molecular weights exceeding filtration limit. Most of the filtered copper is likely to be reabsorbed, because the copper content in urine is normally low. However, copper concentrations in the urine can increase dramatically under disease conditions, as observed in Wilson disease patients (27, 308).

Currently, little is known about renal copper transport and regulation. At least two copper-dependent en-
enzymes, diamine oxidase, involved in the oxidative deamination of histamine and other diamines, and the ferroxidase CP, are produced in kidneys (62, 73). Therefore, renal cells that express these enzymes require Cu-ATPase function for biosynthetic purposes along with the general maintenance of intracellular copper. Which Cu-ATPase plays a key role in the biosynthesis of renal copper-dependent enzymes and how two ATPases contribute to the renal copper homeostasis remains to be characterized. Studies on in-tissue localization of ATP7A and ATP7B provide some insight.

In mice, high copper content was found in the proximal and distal tubules as well as the glomeruli (123, 129). Thus these regions seem to represent the major sites where copper concentration could be regulated through either uptake or export. Inactivation of Cu-ATPase in murine models for either Wilson disease or Menkes disease results in copper accumulation in kidneys (30, 122, 187, 274, 307), with most significant accumulation detected in proximal tubules of the cortex (123, 314). The simplest explanation of this result is that ATP7A and ATP7B function is required in proximal tubules.

This hypothesis has been tested in more recent immunolocalization studies. In kidneys of 10-day-old mice, ATP7A was detected in proximal and distal tubules with very little, if any, staining seen in the glomeruli (82). The staining of ATP7A in the proximal tubules was diffuse, indicative of an intracellular localization and hence, perhaps, a biosynthetic function. In distal tubules, ATP7A was observed at the basolateral membrane, suggesting the role of ATP7A in the transport of copper into circulation (82). Subsequently, the presence of ATP7A mRNA in the proximal tubules and the lack of glomerular staining was confirmed using in situ hybridization in 4-wk-old mice (190). In this latter work, no labeling of ATP7A was detected in the distal tubules, either due to difference in methods, in which either protein (82) or mRNA (190) was detected, or difference in the age of animals. In cultured renal cell lines, ATP7A is detected in both Madin-Darby canine kidney (MDCK) cells, which have a distal origin (81), and opossum kidney (OK) cells, which originate from proximal tubules (unpublished data). Consistent with the role of ATP7A in copper resorption, in polarized MDCK cells ATP7A was shown to traffic to the basolateral membrane in response to high copper (81).

ATP7B mRNA was localized to the glomeruli, and this result correlated with the immunohistochemical localization of the protein (185). Staining of both ATP7B mRNA and protein was also reported in the inner and outer zone of the medulla, which may be the loops of Henle. The inactivation of ATP7B in these regions of nephron may explain massive accumulation of copper-bound metallothionein observed in the outer strip of outer medulla of the ATP7B-deficient LEC rats (141, 200). Different patterns of expression of two Cu-ATPases along with copper accumulation in kidneys of Wilson disease patients (63) also indicate that the lack of ATP7B cannot be fully compensated by ATP7A, and therefore, the roles of ATP7A and ATP7B in renal copper distribution are distinct.

D. Localization and Protein Levels of Cu-ATPases in Placenta Are Regulated by Hormones

Insufficient copper transport to the fetus during pregnancy may produce various abnormalities including embryonic mortality, neonatal growth retardation, and pulmonary and cardiovascular defects. Such deficiency can be dietary, or be caused by inactivation of major components in copper distribution, such as Ctr1 (140, 150), copper chaperone Atox1 (86), or Cu-ATPases. The placenta of both Menkes disease and Wilson disease patients accumulate copper (100, 197). Therefore, it seems likely that ATP7A and ATP7B both have a role in the transfer of copper across the placenta during gestation. This conclusion is supported by studies on expression and localization of ATP7A and ATP7B in human placenta (94). ATP7A and ATP7B were found to be both expressed throughout gestation; however, their localization within the placenta differed.

In general, nutrient uptake from maternal circulation to embryo is mediated by syncytiotrophoblasts and by embryo-derived endothelium cells, which surround the maternal blood vessels within the placenta. ATP7A is localized in the syncytiotrophoblast, the cytotrophoblast, and in the endothelial cells, suggesting that ATP7A may transfer copper from the basolateral surface of the syncytiotrophoblast directly into fetal circulation (94). The ATP7B was also detected in the syncytiotrophoblast, and it was proposed that ATP7B might be present on the apical surface of the placenta and function to return copper from the placenta to the mother, thus preventing accumulation of excess copper in the fetus (94). This hypothesis was recently tested in a cultured cells model (95).

Polarized Jeg-3 cells derived from placental trophoblasts express both ATP7A and ATP7B. In these cells, both Cu-ATPases are localized in the perinuclear compartment and in vesicles, but show little colocalization when costained (95). Further difference between Cu-ATPases was detected following treatment of these cells with hormones or growth factors known to regulate nutrient transport in placenta. Treatment with insulin induced relocalization of ATP7A towards the basolateral membrane and was associated with higher copper transport across this membrane. Earlier, the correlation between levels of ATP7A and copper efflux was observed in another choriocarcinoma cell line, BeWo (226). Altogether, these results are consistent with the proposed role of ATP7A in copper transport from placenta to the fetus.
In contrast, the protein levels of ATP7B were reduced in response to treatment with insulin, and the intracellular localization of ATP7B remained perinuclear. Hormonal treatment also decreased copper efflux across the apical membrane. This result was interpreted as evidence for the role of ATP7B in transport of excess copper from fetus to maternal circulation (95). A more direct support for this hypothesis would be provided by an observation of copper-induced trafficking of ATP7B towards apical membrane, accompanied by increased copper transport across this membrane. Such experiments remain to be done. Additional functions for ATP7B, for example, delivery of copper to CP, which is expressed in placenta (311), should also be considered.

E. Multifaceted Contribution of Cu-ATPases to Central Nervous System Function

Cu-ATPases play an essential role in biochemistry and physiology of the central nervous system (CNS), as evidenced by marked neurological, developmental, and behavioral abnormalities observed in patients lacking either ATP7A or ATP7B. Brain magnetic resonance imaging (MRI) of patients displaying classical Menkes disease often show progressive cerebral atrophy, delayed myelination or even demyelination of white matter, and abnormalities of intracranial vessels (21, 283). Similarly, significant changes in MRI are commonly observed in the basal ganglia of Wilson disease patients. Destruction of white matter and degeneration of cortex vary, although necrosis, spongiform degeneration, and demyelination have been reported (108, 255, 269, 287, 288). These phenotypic manifestations are complex and do not provide obvious insight into specific functions of each Cu-ATPase. The studies attempting to decipher the localization, function, and regulation of Cu-ATPases in the brain are still in their infancy.

Measurements of copper efflux from murine microvascular cells suggested that ATP7A can be involved in copper transfer across the blood-brain barrier (228). Also, the ATP7A mRNA was found highly expressed in the ependymal cells of choroid plexus (109, 138), a structure that regulates the concentration of molecules in the cerebrospinal fluid. Therefore, ATP7A may facilitate copper efflux from the neuropil and/or function with the blood-brain barrier mediating copper entry into the brain. Consistent with this role of ATP7A, the copper levels in the brain and cerebrospinal fluid of Menkes patients are low (132, 162). ATP7A was also detected in numerous other cells within the CNS, including a subset of astrocytes, microglia, oligodendrocytes, tanyocytes, endothelial cells, and neurons (193). The widespread expression of ATP7A indicates that this Cu-ATPase is the major contributor to the maintenance of Cu homeostasis in the CNS. In vitro, ATP7A was detected in rat C6 and PC12 cells, further confirming that both glial and neuronal cells require Cu-ATPase function (227).

Several copper-dependent enzymes play an important role in the CNS; those include PAM, dopamine β-monooxygenase, and CP (98, 218, 268, 298). Expression of ATP7A activity in astrocytes and the olfactory system is consistent with its role in delivery of copper to PAM, which is expressed in these cells (89, 124, 237). ATP7A is also found in myelinating oligodendrocytes ensheathing optic nerve, and copper deficiency in CNS due to ATP7A inactivation is known to cause neuronal demyelination (162). How copper regulates myelination is unclear, although transcription or mRNA stability for myelin components is altered by copper misbalance (162).

Expression of ATP7A in the brain is developmentally regulated (19, 138, 193). It was found to be high in the early postnatal period, reaching maximum in murine neocortex and cerebellum at day 4 after birth (193). Subsequently, in most neuronal cells, the expression of ATP7A decreases, while in the CA2 hippocampal layer the levels of ATP7A are increased (193). Great variability in detecting ATP7A protein and mRNA in Purkinje neurons has been reported (19, 193). Some studies found ATP7A abundant in adult Purkinje cells (189, 193), while other observed it at low levels (109), or greatly downregulated in these cells during development, but present in surrounding Bergmann glia (19). The reason for such differences in ATP7A detection is not entirely clear, but could be related to variations in sample preparation, such as time and intensity of fixing, mouse strains used, and method of detection.

High levels of expression during early brain development are likely to reflect a critical role of ATP7A in CNS at these stages. This conclusion is supported by a striking observation that injection of copper in human Menkes patients or in brindled mice, a murine model of Menkes disease, can prolong survival and alleviate some of the neurological problems (114, 173, 310). Specifically, copper injections can reverse disease-caused frequent tonic seizures and ataxia and partially correct morphological abnormalities of Purkinje neurons (119, 310), although the arborization of the dendrites remains poor. It is interesting that Purkinje neurons also express ATP7B (19), and when copper is delivered (via injections) to the circulation and eventually reaches Purkinje cells, ATP7B appears to at least partially compensate for the lack of ATP7A function.

The localization and function of ATP7B in the brain is much less characterized compared with ATP7A. The ATP7B distribution was analyzed using in situ blotting of 4-wk-old rat brain following direct transfer of native proteins from sectioned tissue to a blotting membrane (244). The ATP7B was detected in neuronal cells of the CA1-CA4 layers of the hippocampus, and this localization was con-
firmed by in situ hybridization. ATP7B was also detected in the glomerular cell layer of the olfactory bulb, and in the granular cell layer of the cerebellum (244). More recent high-resolution studies on ATP7B in adult and developing mouse brain demonstrated that Purkinje neurons are the major site of ATP7B expression in the cerebellum (19). In Purkinje neurons, ATP7B may function in the biosynthetic pathway, delivering copper to ceruloplasmin, which is also expressed in these cells (19). Interestingly, in genetically engineered mice lacking ATP7B, copper delivery to ceruloplasmin is not disrupted (19), suggesting that ATP7A compensates for the lack of ATP7B.

Recent studies suggest that in addition to their roles in maintenance of cytosolic copper concentrations and cofactor delivery to copper-dependent enzymes, Cu-ATPases may have signaling and protective functions in the CNS. In cultured hippocampal, cortical, and olfactory bulb neurons copper acts as a noncompetitive antagonist of N-methyl-D-aspartate (NMDA) receptor (275, 289, 302). Reciprocally, activation of the NMDA receptor was shown to result in trafficking of ATP7A and an ATP7A-dependent vesicular release of copper (253). This novel and interesting link between neuronal activation and copper homeostasis supplied supportive evidence to earlier suggestions that copper may play a role in regulation of neuronal excitability (83, 116).

It was further demonstrated that copper may provide protection of primary hippocampal neurons against NMDA-mediated excitotoxic cell death (254) and that cells lacking ATP7A are more sensitive to NMDA receptor-mediated excitotoxicity. In the mouse models of Menkes disease, insufficient supply of copper to the CNS leads to abnormal structure and function of mitochondria (137, 241), decreased expression of the antiapoptotic protein Bcl-2, and elevated levels of cytochrome c released from mitochondria (241). It seems likely that abnormal mitochondria in mutant mice may have lower capacity to buffer NMDA receptor-gated calcium fluxes (256). Poor calcium buffering capacity and increased apoptosis may explain observed susceptibility of mutant mice to hypoxic/ischemic insults and poor resistance to neuronal injury (254).

Another important function of ATP7A in the CNS is suggested by the peak of ATP7A expression just before synaptogenesis in numerous neuronal subpopulations. In developing neurons, ATP7A is initially present in cell bodies but subsequently found in extending axons. These observations point to a possible role for ATP7A in synapse formation and plasticity (60). It seems particularly interesting that amyloid precursor protein (a causative agent of Alzheimer’s disease) is a copper-binding protein, which is located in neuronal cells predominantly at the synapse (219). How amyloid precursor protein receives its copper is currently unknown, but it is tempting to speculate that ATP7A-mediated copper transport may play an important role in this process. A negative effect of ATP7A overexpression on amyloid precursor protein abundance in cell culture system has recently been reported (22), pointing to a potentially interesting connection between these two proteins.

Altogether, the results described in this section illustrate a high level of integration of copper homeostasis in CNS metabolism and function. The new findings also raise numerous questions about molecular mechanisms that govern ATP7A involvement in various physiological processes in the CNS. They also emphasize challenges of separating direct functional contributions of ATP7A to these processes from indirect consequences of systemic copper deficiency caused by ATP7A inactivation.

F. Developmental Changes in Expression of Cu-ATPases

The developmental changes in the expression of ATP7A in the brain have been discussed in the above section. The data for other tissues are still limited; however, it is clear that ATP7A and ATP7B are regulated in a distinct fashion. ATP7A is widely expressed in both embryonic and adult tissues, and embryonic liver is, so far, the only tissue apart from the brain for which significant developmental regulation of ATP7A was reported (138, 207).

Northern blot analysis demonstrated the presence of ATP7A in the mouse liver at days E17, E19, and P2; however, by P10 and P15, this expression was barely detectable (207). The decline in ATP7A expression shortly after birth is opposite to a change in ATP7B levels, which are increased at this time. It could be that the developmental changes in liver function require Cu-ATPases with somewhat different functional characteristics (for functional comparison of ATP7A and ATP7B, see sect. vB). In addition, ATP7A and ATP7B export copper through different membranes in polarized epithelia (basolateral and apical membranes, respectively). In the fetus, all wastes and presumably excess copper are removed through the blood (via basolateral membrane), while in the postnatal organism, excess copper is excreted via bile. Thus the switch from ATP7A to ATP7B upon liver maturation may reflect the tissue’s need to export excess copper via canalicular (apical) membrane into the bile for eventual removal with feces. Another likely explanation for ATP7A downregulation is that the high level of ATP7A in fetal liver could be due to active fetal hemopoesis and presence of hemopoetic cells, which greatly decline upon birth (207).

The expression of ATP7B during development was found to be regulated in a number of tissues. Iwase et al. (109) demonstrated the presence of ATP7B in the heart and liver at E9.5 and subsequent increased expression in the heart, lung, intestine, nasal epithelia, and liver at E11.5 (109). From E15.5 to E18 expression of ATP7B was de-
rected in the lung, thymus, liver, intestine, and lining of the respiratory tract (109). In the developing intestine, both Cu-ATPases are expressed in the villous epithelium; the role of ATP7B in the intestine is unclear.

Kuo et al. (138) directly compared the expression patterns of the murine Atp7a and Atp7b genes during embryonic development (138). In agreement with other studies, Atp7a mRNA was found throughout the embryo during gestation, whereas the Atp7b mRNA was present in a limited set of tissues, including the liver, heart, CNS, intestine, thymus, and respiratory epithelium (138). The authors proposed that Atp7a might be required for the maintenance of cellular copper homeostasis and the extracellular microenvironment of multiple cell types during development, while ATP7B may perform a more specialized function such as copper delivery to copper enzymes. For example, both ATP7A and ATP7B were found in the embryonic lung; however, ATP7A was localized to the lung parenchyma, whereas ATP7B was highly expressed in the bronchial epithelium. Expression of ATP7B in fetal lung correlates with the expression of CP, which is also detected in bronchial epithelium (126). Coexpression and colocalization of these two proteins suggests that, as in many other tissues, ATP7B plays a key role in the biosynthetic delivery of copper to this enzyme.

IV. GENOMIC AND PROTEIN ORGANIZATION OF HUMAN Cu-ATPases

A. Genomic Organization of ATP7A and ATP7B

The complete exon-intron structure of the Wilson disease gene ATP7B was determined in 1994 (215) and was quickly followed by characterization of the Menkes disease gene ATP7A (54, 282) and the mouse Atp7a gene (37). Presently, the cDNA sequence and genomic information are available for several orthologs of ATP7A and ATP7B as a result of sequencing and characterization of genome of various organisms. This information can be found using Ensemble Genome Browser at http://www.ensembl.org/index.html and the following links for ATP7A http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000165240 and ATP7B http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000123191, respectively.

Both ATP7A and ATP7B are fairly large genes. ATP7A spans ~150 kb of genomic DNA and contains 23 exons with the ATG start codon located in the second exon; the size of the exons varies from 77 to 726 bp (54). The overall structure of ATP7B is similar to that of ATP7A. ATP7B has 21 exons varying from 77 to 1234 bp with the ATG codon for the initiating Met located within exon 1 (215). From exon 5 (exon 3 in ATP7B) to the end of the gene, all of the splice sites in ATP7A occur at exactly the same nucleotide positions as in ATP7B, except for the boundary between exons 17 and 18 (exons 15 and 16 in ATP7B) and a single codon difference at the boundary between exons 4 and 5 of ATP7A (exons 2 and 3 in ATP7B). The only significant difference in the gene structures is observed in the 5'-region encoding the NH2-terminal regulatory metal binding domains (for protein structure, see Figs. 3 and 4). In ATP7B, the first four of six metal binding domains (MBDs) are encoded in one large exon (exon 2), while in ATP7A, the sequence corresponding to the MBDs1–4 is spread over three exons. At the protein level, this region shows considerable sequence diversity between ATP7A and ATP7B and could be responsible for different functional characteristics of ATP7A and ATP7B and/or their trafficking behavior in polarized cells (see sects. V and VI for details).

B. General Architecture of Cu-ATPases and the Functional Roles of Domains

ATP7A and ATP7B encode large membrane proteins with significant primary sequence homology (50–60% identity). In the past several years considerable progress has been made in analyzing the biochemical properties of these two proteins (for recent reviews on structure and function of Cu-ATPases, see Refs. 176, 279, 291). The studies have established that ATP7A and ATP7B belong to the large family of P-type ATPases and have identified several functional domains in their structure (Fig. 4A). It has also become apparent that ATP7A, ATP7B, and their orthologs form a separate subgroup within the P-type ATPase family (P1B-ATPases), which has distinct structural and mechanistic characteristics.

Cu-ATPases transport copper from the cytosol across cellular membranes using the energy of ATP hydrolysis. This process involves specific recognition of copper, delivery of copper to the membrane portion of the transporter with subsequent release at the other side of the membrane, as well as binding and hydrolysis of ATP (Fig. 4B). The central step in the catalytic cycle is the transfer of γ-phosphate from ATP to the invariant Asp residue in the DKTG motif (Fig. 4, B and C) with formation of a transient phosphorylated intermediate. The prerequisite for this reaction is the binding of copper to the sites within the membrane portion of the enzyme, while the release of copper from these sites stimulates dephosphorylation (Fig. 4B). The structural organization of Cu-ATPases reflects the need to accommodate and couple these reactions.

The copper translocation pathway is located in the transmembrane portion of Cu-ATPases, which is composed of eight transmembrane segments (TMS) (Fig. 4). The highly conserved CPC sequence in TMS6 (Figs. 3 and 4) is one of the signature motifs characterizing Cu-ATPases
and ATPases involved in transport of Zn, Cd, Ni, Ag, and Pb, which together form the P1B subfamily (9). Recently, four amino acid residues in transmembrane segments 7 and 8 of the bacterial Cu-ATPase, CopA, were identified as being required for copper binding (171). These residues are conserved in the primary structure of P1B-ATPases involved in the transport of Cu(I). Along with Cys residues of the CPC motif, they are likely to form copper binding site(s) within the membrane portion; in human Cu-ATPases, the corresponding residues are Y1365, N1366, Met1393, and Ser1396 in ATP7A and Y1331, N1332, Met1359, and Ser1362 in ATP7B (Fig. 3). All other functional domains of Cu-ATPases are cytosolic.

1. The NH2-terminal copper-binding domain regulates Cu-ATPase activity

The NH2-terminal metal-binding motifs are highlighted in yellow. The positions of exons are indicated by alternating colors (blue and black); the residues encoded by two exons are in italic. Residues invariant in all P-type ATPases are red; residues that are conserved in Cu-ATPases and form the ATP-binding site are in purple. The recognition sequence for binding PDZ domain proteins, DTAL, at the COOH-terminal end of ATP7B is italicized.

FIG. 3. Primary structure of ATP7A and ATP7B. The NH2-terminal metal-binding motifs are highlighted in yellow. The positions of exons are indicated by alternating colors (blue and black); the residues encoded by two exons are in italic. Residues invariant in all P-type ATPases are red; residues that are conserved in Cu-ATPases and form the ATP-binding site are in purple. The recognition sequence for binding PDZ domain proteins, DTAL, at the COOH-terminal end of ATP7B is italicized.
HCxxCxxxIE (Figs. 3 and 4). Each of these repeats forms a subdomain with a single metal-binding site, i.e., the total stoichiometry is six copper ions per NH2-terminal domain (43, 51, 112, 167, 305). Copper binds in the reduced Cu(I) form, and the two Cys residues in the metal-binding motif CxxC are the only copper-coordinating ligands (50, 231–233). In vitro, and perhaps in vivo, other metals such as zinc (52) or lead (229) can bind to the NH2-terminal domain; however, the functional consequences and physiological significance of zinc or lead binding remain to be determined.

Structural information on individual MBDs of ATP7A has been obtained by several investigators using NMR (13, 15, 18, 49, 76, 113). These studies revealed compactly folded “ferredoxin”-like structures with a \( \beta_{1}\alpha_{3}\beta_{3}\alpha_{3} \)-fold (Fig. 5). The copper-binding Cys residues of the CxxC motif are located in the \( \beta_{1}\alpha_{3} \)-loop and the NH2-terminal portion of the first \( \alpha_{3} \)-helix and, in individual MBDs, are exposed at the protein surface. The conserved Met, Leu, and Phe (Pro in MBD3) residues located in spatial proximity to the CxxC motif (Fig. 5) form a hydrophobic core, which is likely to stabilize the metal-protein complex (10, 76).

Structural and molecular modeling studies of individual MBDs have shown that the distribution of charges on the surface of MBDs differ significantly (for review, see Ref. 11). This difference may be necessary for complementary inter-MBD interactions during folding of the full-length NH2-terminal domain. That such interdomain interaction can be significant is illustrated by the recent structure of the NH2-terminal domain of bacterial Cu-ATPase CopA, which has two metal-binding repeats (17). It has also been suggested that electrostatic interactions of oppositely charged surfaces represent an important step in recognition of copper-chaperone Atox1 by MBDs and copper transfer (304), although recent data indicate that the major role in this process belongs to the metal-coordinating cysteines (14) (see more on Atox1 and transfer mechanism in sect. V).

Extensive mutational analysis and deletion studies were carried out by several laboratories to better understand the functional role of the multiple NH2-terminal metal binding sites (34, 70, 106, 176, 265, 296). One report suggested that all NH2-terminal MBDs in ATP7A can be mutated without significant loss of copper transport function (296). In this early work, the negative controls did not include a catalytically inactive mutant of Asp1044, and a background copper binding might have been underestimated. More recent studies provide convincing demon.
stration that mutation of all six NH₂-terminal MBDs in ATP7B does not alter protein expression, but disrupts ATP7B function (34). This conclusion agrees with the results of several similar studies of ATP7A and ATP7B mutants using yeast complementation system (for example, Refs. 70, 176, 265).

It is now thought that two sites closest to the membrane (in MBD5 and MBD6) are important for the functional activity of Cu-ATPases. This property distinguishes human Cu-ATPases from their bacterial counterparts, which are less dependent on the presence of functional NH₂-terminal MBDs (64, 169, 238). In ATP7A and ATP7B, at least one of these sites should be functional (i.e., able to bind copper) in order for copper transport to occur in a cell. Mutations of copper-coordinating cysteines in either MBD5 or MBD6 of ATP7B alter the apparent affinity of intramembrane binding site(s) for copper (106). This observation suggests that in nonmutated Cu-ATPases, the initial binding of copper to MBD5,6 may stabilize protein conformation that favors subsequent copper binding to the transmembrane portion of the transporter (106). A cross-talk between the NH₂-terminal MBD and the transport sites was also suggested based on studies in bacterial ATPase CopA (238). Whether the NH₂-terminal MBD5 and MBD6 only control enzyme conformation or also donate copper to the transport site remains to be determined.

The first four metal binding sites in MBD1–4 are characteristic of mammalian Cu-ATPases and have a regulatory function. Deletion of the region containing these four sites has no inhibitory effect on Cu-ATPases (106, 176) and does not alter the affinity of the intramembrane transport sites, at least in ATP7B (106). Instead, the deletion of MBDs1–4 facilitates binding and hydrolysis of ATP, suggesting that these additional NH₂-terminal sites, when present, may play an autoinhibitory role (106). This conclusion is supported by studies showing copper-dependent interactions between the NH₂-terminal domain and the ATP-binding domain of ATP7B. Specifically, binding of copper to MBDs in the NH₂-terminal domain was shown to weaken interactions with the ATP-binding domain and result in an increased affinity of the latter domain for ATP (278). Thus it seems likely that one of the roles of the NH₂-terminal MBDs is to modulate enzymatic activity in response to changes in their copper occupancy. It is also possible that copper binding and consequent changes in domain-domain interactions allow Cu-ATPases to adopt specific conformations and/or interact with proteins necessary for the intracellular trafficking of these transporters (for details on trafficking, see sect. vi).

2. ATP-binding domain of Cu-ATPases has distinct nucleotide-coordination environment

By analogy with other P-type ATPases, the catalytic activity of Cu-ATPases (the binding and hydrolysis of ATP) is most likely mediated through coordinated action of the A-domain and the ATP-binding domain (Fig. 4). The latter consists of two portions: the P-domain that includes the site of catalytic phosphorylation and the signature motifs for the P-type ATPases (DKTG, TGDN, GDGxND) and the N (nucleotide binding)-domain (Figs. 4 and 6). The sequence similarity of the P-domains of Cu-ATPases and SERCA Ca²⁺-ATPase was utilized to produce a structural model of the ATP7B P-domain (59) (Fig. 6).

In contrast, the N-domain of Cu-ATPases has little primary sequence homology to equivalent domains of other P-type ATPases. Despite this lack of homology, the three-dimensional fold of the N-domain is very similar to the corresponding domains of other P-type ATPases as indicated by recent high-resolution structures of the ATP7B N-domain (55) and the ATP-binding domain of bacterial Cu-ATPase CopA (55). At the same time, the set of residues involved in coordination of ATP differ between Cu-ATPase and non-P₁B-ATPases. Previous sequence alignments revealed several invariant residues in the N-domain of various Cu-ATPases. In ATP7B these residues are E1064, H1069, G1099, G1101, and G1149; in ATP7A these residues are E1081, H1086, G1126, G1128, and G1183 (Figs. 3 and 6). The nucleotide-binding site in the ATP7B N-domain was mapped by NMR using chemi-
Some experiments demonstrated that all the invariant residues are located in the ATP-binding site (Fig. 6). The G1101 and G1099 are close to the α- and β-phosphates of ATP and may contribute to the tight binding of ATP by the N-domain; the ribose is located in a pocket created by G1149. The adenine moiety is in close proximity to the H1069 imidazole ring and is surrounded by hydrophobic side chains I1180 and I1102. The important role of invariant E1064 in ATP binding was demonstrated by site-directed mutagenesis (186); however, specific function of this residue in nucleotide coordination remains clear. Overall, the solution structure revealed distinct features of the nucleotide-binding environment of Cu-ATPases compared with other P-type ATPases (55). This observation raises a possibility of generating small molecule inhibitors and modulators that may selectively target Cu-ATPases in vitro and in vivo. The need for such small molecule modulators stems from recent observations suggesting the potential role of Cu-ATPases in cancer cell resistance to platinum-based chemotherapeutic drugs (117, 133, 192, 245, 246).

3. Roles of the A-domain and the COOH terminus

The A-domain is located between TMS4 and TMS5. It contains the TGE sequence motif (Fig. 3), which is essential for enzymatic function of P-type ATPases. In the Ca$^{2+}$-ATPase, the Glu residue in this motif is required for the phosphatase step of the catalytic cycle (dephosphorylation of the intermediate formed during ATP hydrolysis) (40). Consistent with this role, a TGE→AAA mutation in the A-domain of ATP7A results in hyperphosphorylated protein (214). Several other mutations in this region of ATP7A were linked to the Menkes disease phenotype, in agreement with the important role of the A-domain in protein function and structure (for example, see Ref. 183).

With the exception of the invariant TGE motif, the primary structure of the A-domain of Cu-ATPases has little similarity to other P-type pumps. Nevertheless, structure prediction algorithms (at http://www.predictprotein.org) recognize the fold of the V$^{901}$-K$^{987}$ of ATP7A as very similar to that of the A-domain of the SERCA Ca$^{2+}$-ATPase. Thus, in a similar way to the N-domain, the overall architecture of this key functional domain of Cu-ATPases is preserved in the absence of primary sequence conservation. This conclusion has been directly confirmed by recent high-resolution structure of the A-domain from bacterial Cu-transporting ATPase CopA (249). Modeling and mutation mapping studies of ATP7A recently performed by Moller et al. (183) further highlighted the functional significance of the A-domain and helped to explain inactivating effects of various Menkes disease mutations.

No structural information is available on the COOH-terminal regions of human Cu-ATPases. The COOH-terminal tails are fairly long (80–100 residues) and contain conserved dileucine (ATP7A) and trileucine motifs (ATP7B), which are required for retrieval of the transporters from the plasma membrane and vesicles (176, 178, 210). Mutations resulting in deletions in the COOH-terminal region were shown to be deleterious for protein stability (102) and to be associated with the disease phenotype (168). In a recent study, Hsi et al. (102) demonstrated that although the COOH terminus of ATP7B is necessary for protein stability, near wild-type levels of ATP7B activity can be detected when 60 amino acid residues are truncated and only one-third of the COOH-terminus is present.

The COOH terminus of ATP7A also contains a PDZ binding motif D$^{1467}$TAL$^{1500}$ (Fig. 3), which was shown to play a role in the targeting and/or retention of ATP7A at the basolateral surface in polarized MDCK cells (81). This motif is located within a 15-amino acid residue segment that interacts with AIPP1 (ATPase-interacting PDZ protein), a small protein with a single PDZ domain (261). The functional consequences of these interactions have not yet been characterized.

C. Alternative Splicing and Protein Variants of ATP7A and ATP7B

Alternative gene splicing is a common mechanism to generate protein products with distinct functional characteristics for finely tuned regulation of cell metabolism. This important mechanism of increasing diversity of a proteome remains greatly understudied in the case of copper transporters, despite the fact that it may hold important keys to a better understanding of great phenotypic diversity observed in patients with mutations in ATP7A and ATP7B. As described below, for both ATP7A and ATP7B many alternate spliced products have been detected; however, remarkably little is known about their functional significance.

1. Splicing variants of ATP7B mRNA

Alternate splicing of ATP7B was first demonstrated in the brain and liver (215). RT-PCR experiments performed on poly(A) RNA isolated from these tissues demonstrated that the most abundant liver transcript contains all 21 exons of ATP7B found in the genomic DNA. The existence of an alternative 22nd exon has been suggested for kidney ATP7B (31); to generate an mRNA containing this exon, the splicing would have to occur within exon 21. The brain, however, appears to contain many splice variants. Curiously, the transcripts include those which are unlikely to produce functional Cu-ATPase. For example, mRNA with exon 17 skipped would lack the sequence encoding the ATP-binding site, while skipping exons 6, 7,
and 8 would delete the predicted TMS1–4. Similarly, splicing out exon 13 would eliminate TMS6, which contains the conserved copper-binding motif CPC. Consequently, the physiological significance of these mRNA variants remains uncertain.

Expression of the recombinant ATP7B variant that lack exons 6, 7, 8, and 12 in HTB9 cells revealed that this truncated protein was dispersed throughout the cytosol in contrast to the TGN location characteristic of the full-sized ATP7B (312). It seems likely that this ATP7B variant was misfolded and nonfunctional as a copper transporter; however, one cannot exclude the possibility that alternatively spliced ATP7B mRNA or the corresponding proteins may perform regulatory functions in the cell.

2. PINA

A very interesting alternative transcript of ATP7B, PINA, is produced in the pineal gland (hence the name, pineal ATPase) and in retina (25). This product is generated from an intronic promoter upstream of exon 9 in a process that results in two transcripts of 3.5 and 4.3 kb (155). PINA expression shows a dramatic diurnal rhythm in both pineal gland and retina, with a 100-fold greater expression in the retina at night than in the day (25). When expressed, PINA mRNA is detected throughout the pineal gland; in the developing eye, it is found within the retinal pigment epithelium layer, the developing ciliary body, and at later stages, in a subset of photoreceptor cells. In adult eyes, PINA is expressed in the region of the outer nuclear layer where cone cells reside.

The function of PINA is not clear. At the protein level, PINA is identical to ATP7B except it lacks all six NH2-terminal MBDs and the first four transmembrane domains. Despite the lack of these important structural elements, PINA appears to have some copper transport activity when tested in a yeast complementation assay (25). The physiological role of PINA is very intriguing; unfortunately, since the initial studies in 1998 and 1999, no further reports on this Atp7b variant have been published. Recent generation of a rat strain in which PINA is defective may help to produce more information regarding the physiological role of this protein (4).

3. Alternative splicing of ATP7A

Alternative splicing of ATP7A has so far been associated mainly with Menkes disease and OHS, a milder manifestation of Menkes disease. In some cases, the mutations in either the splice donor or acceptor sites were shown to cause defects in the efficiency of splicing and reduced levels of otherwise normal mRNA (153); in others, the splice variants with altered mRNA structure were detected. In one such product, the 1.9-kb cDNA had an open reading frame for a protein that lacked exons 3–15 (encoding the NH2-terminal half of ATP7A including 5 MBDs and 6 of 8 transmembrane segments), but still contained sequences for TMS7 and TMS8, one NH2-terminal MBD, and the ATP-binding domain (153). Since the variant lacks most of the transmembrane domain and the A-domain, it is unlikely that the corresponding protein, if produced, can export copper from cells. The authors proposed that this protein could be cytosolic and may have a specific copper trafficking function (153).

Kaler et al. (115) showed that in some patients with OHS, the A>T mutation at a splice site of intron 10 results in an inframe deletion of exon 10, which encodes TMS3 and TMS4 (115). A small amount of this product was also observed in normal individuals where it had a diffuse ER-like staining and was shown to colocalize with the ER marker BIP (225). It was therefore hypothesized that in ATP7A, the transmembrane segments TMS3 or TMS4 play a role in the targeting of ATP7A (71).

This hypothesis was tested using a chimera between the plasma membrane protein CD8 and TMS3 of ATP7A; the chimera protein was shown to partially colocalize with the TGN marker TGN46 (71). Since CD8 by itself traffics to the plasma membrane, alteration of the CD8 chimera localization pointed to the existence of a TGN targeting/retention signal in TMS3. This observation has raised the possibility that alternative splicing in this region may control the abundance/delivery of ATP7A to the Golgi. Interestingly, exon 8 containing TMS3 and TMS4 of the Wilson disease gene (ATP7B) was also shown to be alternatively spliced (215), but intracellular localization and function of this variant have not been studied.

D. Promoters and Transcriptional Regulation of ATP7A and ATP7B

The expression of ATP7A and ATP7B is differentially regulated in several tissues during development (see sect. mE), and recently, the first steps have been made towards dissecting the underlying mechanisms. The promoter regions of ATP7A and ATP7B have been mapped to 1.3- to 2.2-kb segments of the genomic DNA upstream of the coding sequence and the first information on promoter organization began to emerge (97, 154, 166, 198, 199). Oh et al. (199) identified a region ~1.3 kb upstream of the 5’-flanking region of the ATP7B gene, which when analyzed in a reporter assay had high levels of luciferase activity in HepG2 cells. The region contained four metal response elements (MREs), six MRE-like sequences usually found in metallothionein genes, and several putative regulatory elements such as Sp1, AP-1, AP-2, and E-box, but lacked a TATA box. The transcription start site was located 335 bp upstream of the translation initiation site (199). Similar studies of the 5’-UTR of ATP7B were carried out by Loudianos et al. (166). These authors sequenced a 1,228-bp segment 5’ to coding region and iden-
MREa in the promoter region of ATP7B plays a major role in transcriptional activation. MREa was found to bind 70- and 82-kDa proteins that were purified using avidin-biotin affinity chromatography and identified as Ku-related proteins. Subsequent functional studies produced evidence that the Ku-80 subunit is required for constitutive expression of the ATP7B gene (198).

To identify the regulatory elements required for expression of the pineal isoform of ATP7B (PINA; see sect. n/D2) Li et al. (155) characterized sequences upstream of the rat PINA gene and identified a cis-acting element that was recognized by a pineal/retina-specific nuclear factor. This pineal regulatory element (PIRE) has a consensus of TAATC/T and is present in six copies in the 5′- regulatory region of the PINA gene. PIRE binds a retina-specific protein, cone rod homeobox (CRX), which transactivates PIRE-reporter constructs (155).

V. TRANSPORT MECHANISM OF HUMAN Cu-ATPases

A. Heterologous Expression of Cu-ATPases and Functional Assays

The functional activity of ATP7A and ATP7B can be evaluated using a convenient yeast complementation assay, described in detail in section n/B. The assay utilizes a strain of S. cerevisiae (∆ccc2) in which the endogenous Cu-ATPase Ccc2p is genetically inactivated (315). Human Cu-ATPases expressed in ∆ccc2 cells compensate for the lack of Ccc2p and restore copper delivery to the secretory pathway and normal cell growth. The assay has been successfully used by a number of investigators and has yielded useful information about transport activity (or the lack of thereof) for various mutants of ATP7A and ATP7B (70, 102, 104, 107, 315). Although relatively rapid and convenient, the complementation assay is not suitable for quantitative and mechanistic characterization of transporters. For example, minimal activity in a well-expressed mutant may be sufficient to provide phenotypic complementation and generate impression that the mutant is “fully active,” even though essential characteristics such as affinity for physiological ligands and/or the rate of turnover could be markedly altered.

Recently, more direct and quantitative assays for measuring catalytic activity of ATP7A and ATP7B have become available. These functional assays are based on a characteristic property of P-type ATPases to form during hydrolysis a transient intermediate phosphorylated at the invariant Asp residue (Figs. 3 and 4; for review, see Ref. 183). The phosphorylated intermediate, although unstable at alkaline pH, can be isolated under acidic conditions, and the phosphoprotein can be visualized on an acidic gel. The effects of various ligands and conditions (copper, ATP, pH) on the level and rate of formation of the phosphorylated intermediate (as well as its decay) can also be evaluated. The assay has been initially used for solubilized immunoprecipitated ATP7A overexpressed in mammalian cells (295). The study demonstrated that ATP7A forms a phosphorylated intermediate and that copper stimulates this reaction (295), thus confirming that ATP7A functions as a P-type ATPase.

Further information was obtained using heterologous expression of ATP7A and ATP7B in insect cells, which proved to be a useful system for the biochemical analysis of human Cu-ATPases (19, 277). In this method, recombinant ATP7A and ATP7B are produced in Sf9 cells following baculovirus-mediated infection. The levels of expression are high for membrane proteins, permitting detailed biochemical characterization of the Cu-ATPases while still in a membrane environment. With the use of this system, the effect of copper on formation of a phosphorylated intermediate was demonstrated (105, 277), and importantly, the catalytic properties of ATP7A and ATP7B were compared directly (19).

B. ATP7A and ATP7B Have Distinct Enzymatic Characteristics

Experiments measuring the ATP dependence of catalytic phosphorylation (formation of phosphorylated intermediate during ATP hydrolysis) showed that the human Cu-ATPases, when compared under identical conditions, have very similar apparent affinity for ATP, ~1 μM (19, 277). At the same time, the apparent affinities of ATP7A and ATP7B for copper are not the same. [It should be noted that in the experiments analyzing the effect of copper on catalytic phosphorylation, one measures an apparent affinity of copper for the intramembrane sites, since binding to this site(s) is required for catalysis.]

ATP7A has a slightly lower copper binding affinity for the intramembrane site with the apparent K_m of 2.5 μM compared with 1 μM for ATP7B (19). This difference is small, and whether or not it is physiologically significant remains to be established. It is important to emphasize that these measurements were carried out using free copper ions, while in a cell the Cu-ATPases are thought to bind copper as a result of transfer from the cytosolic copper chaperone Atox1 (for details on copper transfer, see section vE). It seems likely that the interactions of ATP7A and ATP7B with the copper chaperone and the efficiency of copper transfer may differ for each ATPase and result in somewhat different responses of the two...
transporters to changing intracellular copper. This could be particularly significant in those cells which simultaneously express both ATP7A and ATP7B (see sect. iii). In these cells, the interactions of the Cu-ATPases with Atox1 rather than the affinity of their intramembrane sites for copper may determine to which ATPase copper is preferentially delivered.

The functional studies in insect cells have also revealed significant differences between ATP7A and ATP7B in the rates with which they carry out the partial reactions (19). Measurements of the kinetics of catalytic phosphorylation and dephosphorylation demonstrate that ATP7A performs each of these steps about sixfold faster than ATP7B (19). Relatively fast phosphorylation and dephosphorylation were also reported for ATP7A immunoprecipitated from mammalian cells (295). Thus it seems that ATP7A is a faster transporter (i.e., it has a higher turnover rate), which would allow ATP7A to remove copper from the cell more effectively than ATP7B. It could be that because of these functional characteristics, in many tissues ATP7A and not ATP7B is expressed and is responsible for maintenance of intracellular copper concentration and copper efflux.

C. Transport Activity of Cu-ATPases

While significant progress has been made in characterizing the enzymatic properties of ATP7B and ATP7A, there have been few reports on direct measurements of copper transport by Cu-ATPases. We still do not know the stoichiometry of copper transport, how copper is coordinated within the membrane (although recent studies on bacterial Cu-ATPases provide first clues on copper coordination (171)), and the parameters that affect copper transport rates.

Measurements of copper transport in Sf9 vesicles have been hindered by high endogenous ATP-dependent copper uptake, and this system remains to be optimized for detecting the transport activity of recombinant ATP7A or ATP7B. Currently, only one group has succeeded in directly measuring copper transport by mammalian Cu-ATPases (mostly by ATP7A) using vesicles isolated from yeast or mammalian cells overexpressing the transporter (290, 293, 294, 296). Using this assay, the authors were able to evaluate the ATP-dependent copper transport by wild-type ATP7A and a number of mutants. Interestingly, some differences in the transport characteristics of ATP7A were seen depending on whether the protein was expressed in yeast or mammalian cells (295, 296). These results suggest that the protein or lipid composition of vesicles may affect copper transport by ATP7A. Only one brief report described copper transport by ATP7B in vitro (293), and no direct comparison of copper transport rates, stoichiometry, or ligand dependencies for ATP7A and ATP7B has so far been published. The expression of ATP7A has been achieved in Xenopus oocytes (24); however, copper transport has not been described in this system.

In recent years, great progress has been made in generating large quantities of highly active bacterial copper-, zinc-, and cadmium-transporting ATPases (58, 170, 238, 257, 306) and measuring their ATPase activity. This is not the case for human Cu-ATPases. Recently, the first report describing purification of Glu-Glu-tagged ATP7A from Sf9 cells and functional reconstitution has been published (105). These experiments yielded measurements of maximum ATPase activity for detergent-solubilized ATP7A, which was estimated as ~55 nmol P$_1$/mg protein$^{-1}$·min$^{-1}$ (or ~10 ATP molecules hydrolyzed by each ATPase molecule per minute). The ATP-dependent copper transport for ATP7A reconstituted into soybean asolectin liposomes was about three orders of magnitude lower (44.9 pmol Cu·mg protein$^{-1}$·min$^{-1}$) (105). These unexpectedly low specific activities could be due to absence of essential lipids in these preparations (105). In addition, it is possible that human Cu-ATPases have inherently lower transport rates compared with their bacterial counterparts. This conclusion is also suggested by recent work reporting the purification and reconstitution of a truncated ATP7B lacking the first five metal-binding domains (217). In this study, after reconstitution into liposomes, the protein had a maximal Cu(I)-dependent ATPase activity of 7–8 nmol P$_1$/mg protein$^{-1}$·min$^{-1}$.

1. Factors that may affect copper transport

The available transport data (see above) and analysis of enzymatic properties of recombinant ATP7A and ATP7B suggest that copper transport by overexpressed human Cu-ATPases is also much slower than ion transport by other P-type ATPases such as Ca$^{2+}$-ATPase or Na$^+$/K$^+$.ATPase. This may not be surprising. Unlike the Ca pump or Na pump that are primarily involved in efficient generation and maintenance of ion gradients, human Cu-ATPases transport an ion, which is not required in large quantities and only needs to be supplied at a rate comparable to the rate of biosynthesis of copper-dependent proteins. Currently, in the absence of specific inhibitors of Cu-ATPases, it has been difficult to obtain estimates of endogenous copper transport in tissues and compare it with the activities of the recombinant transporters. Thus it remains uncertain whether the observed slow transport and enzymatic rates reproduce the physiological situation or reflect the absence in heterologous systems of auxiliary proteins and perhaps posttranslational modifications, which may be required to facilitate transport.

In yeast, copper delivery to copper-dependent enzymes is pH and chloride ion dependent (48). Signifi-
cantly, a study by Wang and Weinman (301) demonstrated that ClC-4, an intracellular chloride channel, colocalizes with ATP7B and stimulates copper incorporation into CP, probably by facilitating copper transport by ATP7B, although an effect of chloride directly on CP loading with copper cannot be excluded.

It is not yet certain whether copper oxidation is required for copper exit from the cells and whether the transport of copper by ATP7A and/or ATP7B is coupled to such copper oxidase activity. CP has a significant copper oxidizing activity (263), and it would be interesting to determine the role of this activity in the release of copper from ATP7B. It should also be noted that a fairly complex dependence of copper transport on the presence of reducing reagents has been reported for bacterial copper pumps and ATP7A (170, 236, 238, 290). The reducing reagents were shown to be necessary not only to reduce copper, but also to somehow modulate the transporter (170, 238). High concentrations of reducing reagents could be inhibitory for the Cu-ATPase activity (238), suggesting that appropriate redox environment is critical for the transporter function, or that there are complex interactions between the transporters and -SH containing molecules.

This is likely to be true for human Cu-ATPases as well. For example, we observed that treatment of ATP7B with a copper chelator in the presence of ascorbate results in rapid irreversible inactivation of ATP7B unless another reducing reagent, tris(2-carboxyethyl)phosphine (TCEP), was also present (277). It is intriguing that glutathione, a protein that catalyzes the reduction of disulfide bridges and reverses protein glutathionylation, is an interacting partner for both ATP7A and ATP7B (157). Perhaps copper release from the transporter can result in oxidation of critical Cys residues, and the presence of appropriate reducing reagent or, in a cell, modification with glutathione is essential to protect these residues against oxidation.

2. Transport activity of Cu-ATPases in different cell locations

The dual function of Cu-ATPases in a cell (copper delivery to the secretory pathway and copper export) is achieved through relocation of the transporters from the TGN to vesicles and then eventually to the plasma membrane (Fig. 7, for details see sect. vi). It is assumed that Cu-ATPases transport copper effectively in all these cell locations; however, comparison of Cu-ATPase function in cell compartments is technically difficult and so far has not been carried out. Also, little evidence is available showing that copper export from cells is due to the Cu-ATPases pumping copper directly across the plasma membrane. In fact, recent studies strongly suggest that the major localization and hence function of these transporters is in the intracellular compartments, and their presence at the plasma membrane could be a result of fusion of the ATPase-containing vesicles with the plasma membrane during the exocytosis/recycling process (184) (Fig. 7).

It is worth considering that the environments of Cu-ATPases in the TGN, intracellular vesicles, and at the plasma membrane are quite different, and thus the rates of transport in different cell compartments may vary considerably. In the TGN, where copper transport was convincingly demonstrated using the in-cell tyrosinase assay (85, 213) (see sect. II.B1), the copper released from the Cu-ATPase is taken up by copper-binding proteins, such as tyrosinase, ceruloplasmin, etc. The protein-mediated sequestration of released copper may provide a “copper sink,” decreasing the gradient against which the ATPases work and hence facilitating transport into vesicles. In fact, some aspects of copper transport may be similar to the calcium uptake into intracellular vesicles mediated by SERCA1, a Ca$^{2+}$-transporting P-type ATPase. In vitro, the transport of calcium into vesicles by SERCA1 is potentiated by luminal oxalate, which forms complexes with calcium, and thus decreases the luminal calcium concentration (303). In cells, calcium buffering in the lumen of sarcoplasmic reticulum (SR) is provided by an SR resident calsequestrin and other calcium binding proteins.
For the human Cu-ATPases, such copper-buffering function in the lumen of TGN can be accomplished by secreted copper-binding enzymes.

In contrast, in the intracellular vesicles to which Cu-ATPases relocalize in response to elevated intracellular copper, the pH remains acidic (i.e., favorable for metal release), but specific copper accepting proteins may no longer be present. Thus it is not clear whether in vesicles the ATPases transport copper with any appreciable rate, if at all. It could be that after copper concentration in the TGN lumen reaches a critical level and the Cu-ATPase leaves the TGN, the subsequent function of Cu-ATPases is to target vesicles (already filled with copper in the TGN) to the intracellular machinery that directs these vesicles to the plasma membrane. This coupling between the Cu-ATPase-containing vesicles and cytoskeleton can be mediated through specific adaptor proteins such as Murr1/CommD1 (271) (see sect. viD4). The vesicles would then travel to the plasma membrane, where copper can be exported via vesicle fusion and exocytosis. Measuring copper efflux after repeated treatments of cells with copper may help to test this hypothesis and determine whether or not human Cu-ATPases function in the vesicles.

D. Copper Delivery From Cytosol to the Intramembrane Sites of Cu-ATPase

How ions migrate from the cytosol to intramembrane sites in the protein remains an intriguing and largely unanswered question for most P-type ATPases. In Cu-ATPases, this step of the ion-translocation mechanism seems particularly complex due to the presence of multiple copper-binding sites (Figs. 3 and 4) and the apparent need to keep copper in a protein-bound state. Cu(I), the form of copper that binds to the NH2-terminal domain of Cu-ATPases, the pH remains acidic (i.e., favorable for metal release), but specific copper accepting proteins may no longer be present. Thus it is not clear whether in vesicles the ATPases transport copper with any appreciable rate, if at all. It could be that after copper concentration in the TGN lumen reaches a critical level and the Cu-ATPase leaves the TGN, the subsequent function of Cu-ATPases is to target vesicles (already filled with copper in the TGN) to the intracellular machinery that directs these vesicles to the plasma membrane. This coupling between the Cu-ATPase-containing vesicles and cytoskeleton can be mediated through specific adaptor proteins such as Murr1/CommD1 (271) (see sect. viD4). The vesicles would then travel to the plasma membrane, where copper can be exported via vesicle fusion and exocytosis. Measuring copper efflux after repeated treatments of cells with copper may help to test this hypothesis and determine whether or not human Cu-ATPases function in the vesicles.

E. Atox1-Mediated Transfer of Copper to Cu-ATPases

The protein-mediated distribution of copper from the uptake system (CTR1) to the export system (Cu-ATPases) is a unique feature of copper transport compared with the transport of other ions such as sodium, calcium, or protons. This delivery is carried out by a small cytosolic protein Atox1, which has a striking structural homology to the individual metal-binding domains of Cu-ATPases (103, 234, 304). EXAFS studies demonstrated that in Atox1 copper is coordinated by two sulfurs donated by the Cys residues in the conserved metal-binding site MxCxxC (234) in the environment very similar to that of MBDs. It was proposed that upon Atox1 docking to individual MBDs, the transient intermediate is formed in which the third sulfur ligand is donated by the Cu-ATPase MBD resulting in copper exchange (304). Copper-dependent interaction between Atox1 and MBDs has been observed for both ATP7A and ATP7B (147, 264), and recent NMR studies by Banci et al. (16) demonstrated the formation of a Cys-based protein adduct for MBD2 or MBD4 of ATP7B and Atox1. No stable adduct is formed between MBD2 or MBD5 of ATP7A and Atox1 (16) or between Atox1 and MBD5,6 of ATP7B (1).

The vectorial copper transfer from Atox1 to MBDs can be explained by the fact that MBDs retain copper much more effectively than Atox1 (16, 299). Specifically, experiments comparing the ability of the copper chelator BCS to remove copper from holo-Atox1 compared with holo-MBD2 of ATP7B revealed that a 100-fold excess of BCS is sufficient to remove metal from Atox1 following 15-min incubation, while only 10% of copper is removed from MBD2 under the same conditions (299). Recent detailed NMR studies of MBD2–5 and Atox1 provide a structural explanation of this phenomenon. In Atox1, only minor structural rearrangements take place upon copper binding (7), while binding of copper to a MBD causes more significant structural changes in the protein. In MBD2 of ATP7A, for example, the changes involve an extension of the β1 strand by one residue and α1 helix by five residues (18), which occurs in the immediate vicinity.
of the bound metal and lead to a greater shielding of copper from the aqueous solution compared with Atox1. How and whether copper is then released from this partially hidden state and transferred further is a very interesting question that is still to be addressed.

The loading of the metal-binding sites in the NH₂-terminal domain by Atox1 is likely to proceed through several discrete steps (299, 300) and could be based on a clear preference for Atox1 interaction with some MBDs over the others (16, 147, 299). Copper transfer experiments using purified Atox1 and the recombinant NH₂-terminal domain of ATP7B revealed that in ATP7B, MBD2 is the site to which Atox1 transfers copper preferentially and that the selectivity towards this metal-binding domain is observed only if Atox1 (and not ascorbate or glutathione) is used as a copper donor (299). Mutations of the metal-coordinating cysteines in MBD2 of the full-length ATP7B completely disrupt copper-dependent catalytic phosphorylation of the ATP7B mutant when Atox1 is used as a source of copper (299). Significantly, the ability of “free” copper to stimulate catalytic phosphorylation of this mutant is not changed. This result indicates that the intramembrane sites remain fully functional and accessible, if MBD2 is bypassed.

On the basis of stronger protein-protein interactions between isolated MBD4 and Atox1 in solution, it was proposed that MBD4 rather than MBD2 is a preferential site for copper transfer from Atox1 (1). However, MBD4 is not functional in rodent ATP7B (276), and therefore, the initial transfer of copper from Atox1 to MBD4 is unlikely to be an essential step of ATP7B function. Nevertheless, all current data can be easily accommodated by the following model. The transfer of copper from Atox1 to MBD2 is the first step that allows delivery of copper from the cytosol to the intramembrane sites of the transporter. Binding of a single to MBD2 is accompanied by a conformational change in the NH₂-terminal domain (299, 300) and serves as a molecular switch initiating copper delivery to the intramembrane sites. Specifically, the change allows access of Atox1 to the other metal-binding sites, such as MBD4, and further transfer of copper to, perhaps, MBD5 and/or MBD6 (see above). This model is also consistent with data showing that MBD4 can transfer copper to MBD5 and MBD6 (1), while Atox1 does not. Copper can then migrate towards the transport site either from MBD6 or directly from copper chaperone (see below) via a series of copper-coordinating residues (for example, a chain of Met residues in the first transmembrane hairpin). The high-resolution structure of the full-length Cu-ATPase, when available, will be fascinating and will provide essential clues to the precise copper-translocation pathway.

It seems likely that under low copper conditions the NH₂-terminal domain remains partially metallated with only a fraction of sites occupied at the time. When copper concentration is increasing, all MBDs become occupied.

VI. REGULATION OF Cu-ATPase FUNCTION THROUGH INTRACELLULAR TRAFFICKING

The dual role of ATP7A and ATP7B in delivering copper to the biosynthetic pathway and exporting copper from the cell is achieved via regulated targeting of Cu-ATPases to different cellular membranes (Fig. 7). The original studies from Petris et al. (212) and subsequent work from several laboratories (for details see below) provided convincing evidence that Cu-ATPases have distinct intracellular localizations depending on whether or not cells were exposed to elevated copper. It has been postulated that the copper-induced relocalization of the transporter is necessary to export excess copper from the cell.

In recent years, it has become increasingly clear that the targeting and trafficking of Cu-ATPases can be influenced not only by changes in copper concentration but also by such factors as the transporters’ catalytic activity, presence of specific sequence elements in the ATPase structure, posttranslational modification, and possibly by various protein modulators (see below for details). Furthermore, trafficking of Cu-ATPases in response to treatment with hormones, such as prolactin, or NMDA-receptor activation has been reported (121, 253), indicating additional levels of cell control over the Cu-ATPase function. The mechanistic picture on how the intracellular localization of Cu-ATPases is regulated is yet to emerge; nevertheless, the current data allow speculation about several possible mechanisms that are discussed in the following sections.
A. Copper-Dependent Trafficking of ATP7A

Most of what we know about trafficking of human Cu-ATPases is based on studies of ATP7A. There are certain similarities in trafficking behavior of ATP7A and ATP7B (for review, see Ref. 176). However, in polarized cells, these two proteins reallocate to different membranes (basolateral and apical, respectively), and specific molecular machinery that govern their reallocation can be quite different. The majority of experiments on ATP7A have used cells either transiently or stably transfected with ATP7A cDNA, although the localization of endogenous ATP7A in CHO cells and in human skin fibroblasts was also investigated (6, 53). Under basal growth conditions, ATP7A was repeatedly shown to have perinuclear localization, which overlapped with a Golgi marker α-mannosidase II (53). Treatment with brefeldin A further delineated ATP7A localization as being consistent with the TGN. Brefeldin A causes the TGN to collapse around the microtubule organizing center (MTOC), which appears as tight perinuclear staining (152), while the rest of the Golgi redistributes into the ER in a diffuse pattern. In cells treated with brefeldin A, ATP7A staining is perinuclear, as expected for TGN localization (53).

In the TGN, ATP7A is active and transports copper for incorporation into copper-dependent enzymes, such as tyrosinase and PAM (see sect. \(\mu B3\)). The TGN localization, however, is not static; rather, it reflects the steady-state distribution of ATP7A, which continuously cycles between the TGN, endosomal vesicles (202), and the plasma membrane (Fig. 7). The available data suggest correlation between the intracellular localization of ATP7A and its primary function in the cell. For example, in pituitary endocrine cells, where ATP7A is required for biosynthetic incorporation of copper into PAM, ATP7A is predominantly located in the TGN (61). In contrast, in the kidney, the major role of ATP7A is likely to be in copper reabsorption into the blood, and in this tissue ATP7A immunostaining is diffuse consistent with vesicular localization rather than TGN (163). The intracellular localization of ATP7A can also be controlled by the metabolic state/needs of cells and may differ at different stages of development. Such dependence has been demonstrated in recent studies of ATP7A in mammary gland cells (2, 121) (see sect. \(\mu B3\)).

1. Physiological relevance of copper-dependent relocalization of Cu-ATPases

The initial observation of copper-dependent trafficking of ATP7A from Golgi network to vesicles in close proximity to the plasma membrane was verified by immunogold staining of CHO-K1 cells and electron microscopy (142) and was subsequently confirmed by several groups using ATP7A cDNA transfected in a variety of cell types (42, 53, 79, 81, 202). Many experiments on Cu-ATPase trafficking have utilized 189–200 \(\mu M\) copper, which is much higher than the physiological concentration of copper in a serum (total copper is \(~15–20\) \(\mu M\)). Such a high concentration is not necessary to stimulate relocation of ATP7A in cell culture systems. The trafficking is detectable in cells treated with 10 \(\mu M\) copper or lower, particularly if colocalization with organelle markers is performed (see, for example, Refs. 87, 196, 212). However, high concentrations are required to detect ATP7A at the plasma membrane [at lower concentrations ATP7A is localized predominantly in vesicles as observed in tissues (184)].

Recent studies in transgenic mice overexpressing ATP7A have provided evidence for the physiological relevance of copper-induced trafficking by showing that ATP7A relocalization occurs in tissues (184). Perfusion of an isolated segment of the jejunum with a solution containing different concentrations of copper chloride induces the intracellular redistribution of ATP7A towards vesicular compartment adjacent to the basolateral membrane. In contrast to earlier cell culture experiments, plasma membrane staining of ATP7A was not observed. This observation led to the suggestion that the transport of copper enterocytes into the blood “may involve ATP7A pumping copper into a vesicular compartment followed by exocytosis to release the copper, rather than direct pumping of copper across the basolateral membrane” (184).

The observations suggesting that copper export occurs via vesicles-mediated exocytosis raise several questions. For example, why is this mechanism preferred over a more commonly employed direct translocation of ion across plasma membrane by the transporter? Is there a mechanistic difference between eukaryotic and bacterial Cu-ATPases, as the latter function at the plasma membrane? Why is exposure to very high levels of copper necessary to detect ATP7A at the plasma membrane, while only 1–10 \(\mu M\) is sufficient for relocalization? Answering these questions may provide useful mechanistic insights. For example, the potential role of luminal pH in copper release has been already discussed (see sect. \(\nu C2\)). If a relatively low luminal pH is required for Cu-ATPase function, then plasma membrane localization would be unfavorable for copper transport, while vesicular compartments would provide an appropriate environment. From this point of view, it is interesting that bacterial cells (unlike mammalian cells) maintain a proton gradient at their cellular membrane, and protonation of extracellular/luminal residues may contribute to copper transport mechanism by either bacterial or human copper pumps.

2. Does copper stimulate forward trafficking or inhibit endocytosis?

A copper-induced change in the intracellular localization could be caused by copper either stimulating the exit
of Cu-ATPase from the TGN or inhibiting endocytosis and subsequent return to the TGN. These two possibilities were examined in experiments using a myc-tagged ATP7A, with the epitope inserted into the extracellular loop between TMS1 and TMS2 (211). Incubation of cells expressing this construct with the anti-myc antibody results in the accumulation of the endocytosed antibody in the TGN region. This occurs as a result of ATP7A constitutive cycling from the TGN to the plasma membrane, where the epitope is exposed to antibody binding, and then returns to the TGN by endocytosis. In elevated copper, the antibody internalization can still be detected (211), suggesting that copper stimulates trafficking rather than prevents endocytosis of ATP7A.

This conclusion was further supported by experiments using the ATP7A-myc tagged mutant in which the COOH-terminal endocytic signal L1485L1486 was inactivated by Ala substitution. This mutation leads to impaired endocytosis of ATP7A and trapping protein at the plasma membrane. As a result, in basal copper conditions the ATP7A mutant is detected in both the TGN and plasma membrane. In high copper, the staining of the ATP7A mutant at the TGN completely disappears, confirming that copper stimulated the TGN exit (211). How elevated copper induces forward trafficking of the Cu-ATPase is unknown; several possible mechanisms have been proposed and are discussed in sections below.

While the above experiments point to a stimulatory effect of copper on forward trafficking, the exact influence of copper on endocytosis is less clear. Recent analysis of ATP7A trafficking using surface biotinylation sheds some light onto this issue (203). Pase et al. (203) demonstrated that 100 μM copper increases the total amount of ATP7A at the plasma membrane by only two-fold, although under these conditions the majority of ATP7A leaves the TGN. This result is highly suggestive of the transient presence of ATP7A at the plasma due to a rapid endocytosis. Further increase of the copper concentration up to 500 μM speeds up the forward trafficking (the t1/2 for plasma membrane delivery decreases ~2-fold) and also increases the steady-state amount of ATP7A at the cell surface (further 2- to 2.5-fold increase compared with 100 μM copper).

These results are best explained by simultaneous and opposite effects of copper, i.e., stimulation of ATP7A forward trafficking and inhibition of endocytosis. It was shown that time (t1/2) for reaching equilibrium can be equated with the rates of endocytosis (203). Nevertheless, these studies have provided the first demonstration of a rapidly recycling pool of ATP7A. Such pool may play an important role in maintaining intracellular copper under high copper condition, since vesicles can be refilled with copper in close proximity to the membrane and thus provide more efficient export of excess copper. The vesicular pool in close proximity to the plasma membrane is also best suited to provide rapid copper release in response to, for example, calcium signaling (253).

Studies of PDZ domains and their effects on ligand-induced trafficking show that these domains can be important for plasma membrane retention of proteins in polarized cells (208). The presence of the PDZ domain in protein structure can delay endocytosis, and this effect could be highly compartmentalized, i.e., it can take place in only a subset of clathrin-coated pits (224). Therefore, a PDZ domain containing proteins can be found in two populations: one arrested at the plasma membrane and the other removed into the recycling pool (224). ATP7A has been shown to contain a PDZ motif and to interact with a specific PDZ protein AIP1 (261). The deletion of PDZ motif DTAL results in loss of polarized targeting of ATP7A in agreement with the possible role of this motif in the retention at the basolateral membrane. It would be interesting to quantitatively evaluate the ATP7A endocytic and recycling rates in polarized cells in the presence and absence of the AIP1 to better understand factors that control ATP7A trafficking.

B. Localization and Trafficking of ATP7B

 Trafficking of endogenous ATP7B has been studied in primary hepatocytes, as well as in cultured hepatic cells, HepG2, and Hep3B, where ATP7B is most abundantly expressed and ATP7A is not detected. In basal medium, ATP7B localizes to a trans-Golgi compartment, which also contains syntaxin 6 (85). Immunostaining of ATP7B in this location is particularly compact when HepG2 cells are treated with 40 μM BCS, a copper chelator (104, 239, 284, 312), while addition of copper results in relocalization of ATP7B (104, 239, 284). Curiously, when HepG2 cells were treated with 200 μM Cu-histidine, no change in the localization of ATP7B was observed (104), suggesting that the response of HepG2 cells may depend on the form of Cu presentation. Administration of copper in vivo showed the redistribution of hepatic ATP7B to vesicles in proximity to the canalicular membrane in agreement with the role of ATP7B in biliary copper excretion (251). In this location, ATP7B is targeted to unique subapical vesicles (85). Upon copper decrease, ATP7B returns to the TGN, in a similar fashion to ATP7A. Al-
though it is likely that ATP7B also cycles via plasma membrane, the conclusive evidence for ATP7B reaching plasma membrane is lacking.

Studies of transfected ATP7B in various cell lines, including CHO-K1 (hamster ovary cells), fibroblasts from Menkes disease patients, HTB9 (bladder) cells, hepatic HUH7 cells, generally agree with observations made for endogenous ATP7B (34, 69, 92, 118, 206, 312). However, the green fluorescent protein (GFP)-tagged version of ATP7B was also found in the late endosome and was insensitive to elevated copper (91–93). This apparent difference in the trafficking behavior of ATP7B could be due to the difference in levels of ATP7B expression in transiently (64–66) and stably transfected cells ATP7B (118), as GFP does not affect targeting of ATP7B in polarized hepatocytes (85).

An interesting recent work describes an induction of trafficking of ATP7B upon treatment of cells with 2 μM cisplatin (118). Cisplatin is a platinum-containing compound frequently used as a chemotherapeutic agent in cancer treatment. Cisplatin-resistant cells have been shown to upregulate the expression of ATP7B (or ATP7A). It is intriguing that such low levels of cisplatin induce ATP7B trafficking. Copper-dependent trafficking of ATP7B can be detected upon exposure to 5–10 μM copper (239, 284); however, typically higher concentrations are used. If the concentration of ligand inducing the ATP7B trafficking is a reflection of its ability to bind to the ATPase, then it would appear that cisplatin binds to ATP7B in a cell as well as copper. A direct comparison of the ATP7B binding/transport of copper and cisplatin could be very interesting.

Several mammalian orthologs of ATP7B have been cloned and characterized, providing a useful source of information for identifying sequence elements critical for ATP7B trafficking. The mouse ortholog mATP7B, which is ~82% homologous to human ATP7B, was expressed in hamster CHO-K1 (143) and human Me32a-T22/2L fibroblasts (144) and localized, as expected, in the perinuclear compartment. Surprisingly, mATP7B showed no trafficking response in Me32a-T22/2L cells, but relocalized to vesicles in CHO-K1 fibroblasts. This result suggests that recognition of ATP7B by the trafficking machinery is species-specific and that sequence differences of ATP7B orthologs may be utilized for dissecting the structural elements required for protein-protein interactions and trafficking (144).

C. Molecular Mechanism of Copper-Dependent Trafficking of Cu-ATPases

Despite significant interest and numerous studies, the events that lead to ATP7A and ATP7B exit from the TGN copper remain unknown. Several possible mechanisms have been discussed in the literature. It has been suggested that the increase in intracellular copper results in saturation of the NH2-terminal domain of ATP7A with copper, resulting in a conformational change of this domain, which in turn serves as a signal for initiation of a trafficking response (50). Another model directly links the catalytic activity of ATP7A to its trafficking, implying that increased intracellular copper raises the pool of active protein, which in turn is recognized as ready for trafficking (176, 214, 294). Lastly, elevated copper was found to increase the kinase-mediated phosphorylation of human Cu-ATPases, suggesting that posttranslational modification and trafficking of Cu-ATPases could be linked (284, 292). The following sections describe supportive evidence that exists for each of these proposals. It should be emphasized that suggested scenarios are not mutually exclusive and that the actual mechanism of copper-dependent protein trafficking is likely to be complex and include elements of each model.

1. Does copper binding to the NH2-terminal domain serve as a signal for trafficking?

The role of the NH2-terminal MBDs in copper-dependent trafficking has been extensively studied. Considerable differences in trafficking behavior of Cu-ATPase mutants were observed depending on the number and location of mutations (for example, compare Refs. 79 and 265). It is possible that mutations in the metal binding sites not only disrupt copper binding by the MBDs but also alter the interdomain interactions and protein conformation of Cu-ATPases. These structural changes would additionally modify the protein’s ability to respond to elevated copper.

It was also shown that conformation of the NH2-terminal domain changes in response to copper binding (50, 299), leading to the suggestion that this conformational change may serve as a signal for copper-dependent relocation of Cu-ATPase (50). However, it has also become clear that the first four NH2-terminal MBDs, while playing an important role in regulating enzyme activity (278), are not essential for trafficking. The segment containing these sites can be mutated/deleted without losing the ability of Cu-ATPase to relocalize in response to elevated copper (34, 85, 265). Whether decreasing the number of the NH2-terminal MBDs alters the sensitivity of Cu-ATPase to intracellular copper has not been established.

Inactivation of all six NH2-terminal copper binding sites does inhibit trafficking of either ATP7A or ATP7B (79). A similar effect was observed for the Cys>Ser mutation in the intramembrane CPC motif that is expected to inactivate copper binding. These substitutions also greatly impair protein function (34, 35, 70), thus making it difficult to separate the effect on copper binding from...
conformational and functional effects. It is interesting that insertion of an additional mutation, $^{875}\text{TGE}^{877}\text{AAA}$, restores the ability of ATP7B with six inactivated MBDs to traffic to vesicles (35). Furthermore, this $^{875}\text{TGE}^{877}\text{AAA}$ mutation allows copper-dependent relocation for ATP7A with the CPC and six MBDs all inactivated (35).

Since this latter mutant has all known copper-coordinating residues inactivated, it was suggested that ATP7B contains additional copper binding site(s) necessary to provide copper-dependent trafficking (35). Where this extra site is located is currently unclear. CPC are not the only intramembrane residues that participate in copper coordination in $\text{P}_{1\text{B}}$-ATPases (57, 171, 306); therefore, one cannot exclude that in the TGE>AAA mutant, these other intramembrane residues reorganized to form a high-affinity copper binding site.

A very interesting study has recently been published by Guo et al. (85) who used polarized hepatic WIF-B cells to investigate trafficking of ATP7B. These authors found that both the copper response and correct apical targeting of ATP7B in elevated copper required the presence of the most N$\text{H}_2$-terminal 63 amino acid residues, even when a large portion of the N$\text{H}_2$-terminal domain, including the first 5 copper binding sites, is deleted (85). These results suggest that important targeting information for ATP7B is contained in this relatively short N$\text{H}_2$-terminal sequence. It is particularly interesting that this segment is absent from the primary structure of ATP7A (see Fig. 3), which traffics to the basolateral membrane. Whether this short segment interacts with cellular trafficking machinery or modulates such interaction via interdomain contacts remains to be established.

2. The link between the catalytic cycle of Cu-ATPases and their trafficking

Current data suggest that the catalytic and transport activity of Cu-ATPases may be linked to their ability to traffic. This hypothesis originated from the experiments on ATP7A constructs mutated in the regions important for ATP7A activity (6). The C1000R or CxC>SxS substitutions in the CPC motif resulted in TGN localization and loss of copper sensitivity (214, 294). Similar results (targeting to TGN, but no response to copper) were observed with mutations of invariant residues in the ATP binding domain, H1086 and D1044, and conserved N1233 in TMS7 (214). The His1086 in ATP7A is equivalent to His1069 of ATP7B, which is the site of the most frequent Wilson disease mutation (33, 36, 68) and is required for high-affinity ATP binding (186, 294) and trafficking (206). D1044E replaces catalytic residue involved in acyl-phosphate formation, while mutation of N1233 mutant affects a putative copper coordinating residue.

Further study, however, revealed that the ability to adopt certain conformations could be more important for trafficking than the transport activity per se. The ATP7A construct containing a disease mutation $L873R$ in the A domain was found to localize to the plasma membrane in both basal and elevated copper (214). A similar result was observed for the $^{875}\text{TGE}^{877}\text{AAA}$ mutation described above. The TGE motif in the A-domain (Fig. 3) is essential for the enzymatic activity of P-type ATPases, and the TGE>AAA mutant is inactive with respect to copper transport, as confirmed by the yeast complementation assay (35, 214). However, the TGE>AAA mutant can undergo catalytic phosphorylation, and the level of catalytic phosphorylation for this mutant is higher compared with control ATP7A. It was proposed that the $^{875}\text{TGE}^{877}\text{AAA}$ mutant is stabilized in a hyperphosphorylated state and that it is in this state that the protein relocates to the plasma membrane even in the absence of elevated copper (214).

This observation is very important as it offers a possible explanation of how trafficking can be triggered in response to elevated copper. As we discussed in section V, catalytic phosphorylation of Cu-ATPases requires binding of copper to the transmembrane site (also see Fig. 4B). Dephosphorylation in turn is associated with the release of copper from the intramembrane site into the lumen. Under basal conditions, the metal ions transported into the lumen of the secretory pathway are picked up by copper-dependent enzymes, and it is likely that no free copper is present in the lumen under these conditions. When copper is elevated, more copper is being pumped into the secretory pathway, and it may eventually exceed the binding capacity of acceptor proteins and reach saturation. In this state, the release of copper from the transporter will be inhibited, and the Cu-ATPase would be stabilized in conformation very similar, if not identical, to that of phosphorylated Ec$_{PCu}$ enzyme, i.e., the state in which the protein is susceptible for interaction with the trafficking machinery.

This hypothesis would predict that 1) the Cu-ATPase variants with lower transport activity would have delayed trafficking response or apparent lack of copper sensitivity and 2) overexpression of the acceptor protein in the secretory pathway may slow down or completely prevent trafficking of the ATPase. A possible example is the H1086Q mutant of ATP7A, which has markedly decreased transport activity and does not traffic in response to treatment with 200 $\mu$M copper, but does relocalize when cells are treated with 300 $\mu$M copper (294).

D. Protein Machinery Involved in Cu-ATPase Trafficking

1. Does ATP7A use a clathrin-mediated pathway for trafficking?

Dileucine motifs have been implicated in clathrin-dependent endocytosis of membrane proteins (152).
Cu-ATPases or lock it in a conformation compatible with other proteins; it may also affect the activity of ATPase phosphorylation; in both Cu-ATPases, the amino acid that in CHO-K1 cells also undergoes a kinase-mediated phosphorylation is a serine residue by phosphoamino acid analysis (285, 292).

ATP7A is phosphorylated was shown to be a serine residue by phosphoamino acid analysis (285, 292). ATP7A expressed in CHO-K1 cells demonstrated that ATP7A is phosphorylated by a kinase and the level of phosphorylation increases following cell exposure to elevated copper (284). The phosphor-ylation of ATP7B is copper specific and reversible, i.e., following decrease in copper concentration ATP7B is de-phosphorylated. Interestingly, the level of phosphorylation correlates with the intracellular location of the copper transporter. A basal level of phosphorylation of ATP7B is observed at the TGN, while in the vesicles ATP7B is hyperphosphorylated (284). ATP7A expressed in CHO-K1 cells also undergoes a kinase-mediated phosphorylation; in both Cu-ATPases, the amino acid that is phosphorylated was shown to be a serine residue by phosphoamino acid analysis (285, 292).

The kinase-mediated phosphorylation of ATP7A and ATP7B may initiate trafficking by facilitating interactions with other proteins; it may also affect the activity of Cu-ATPases or lock it in a conformation compatible with the retention at the plasma membrane/vesicles. Determining the precise role of a kinase-mediated phosphorylation in Cu-ATPase trafficking may also contribute to understanding how signaling via NMDA receptor activation or in response to hormones induces ATP7A relocalization in neuronal and mammary gland cells, respectively (see sect. iii on studies of ATP7A in tissues).

Current information on molecular basis of phosphorylation is still very limited. The copper-induced phosphorylation appears to require the presence of a functional NH2-terminal domain. The pineal isoform of ATP7B, PINA (see sect. ivC2), lacks the NH2-terminal domain and undergoes basal phosphorylation but shows no hyperphosphorylation in response to copper (284). Similarly, the NH2-terminal mutant of ATP7B, G591D, which does not interact with the copper chaperone Atox1 and presumably has lost the ability to bind copper (88), also does not change its level of phosphorylation when copper is elevated. Loss of copper-dependent phosphorylation was also reported for the nontrafficking mMBD1–6 mutant of ATP7A (292). These results may also indicate that copper-dependent phosphorylation takes place upon relocalization of Cu-ATPase to vesicles and serves as an important retention signal.

Protein kinase D (PKD) has been shown to regulate trafficking of some proteins from the TGN to the plasma membrane (313). To examine the possible involvement of PKD in ATP7A trafficking, Cobbold et al. (42) cotrans- fected the dominant negative PKD variant PKD-K618N and ATP7A into HeLa cells. PKD-K618N did not inhibit the trafficking of ATP7A (42), ruling out PKD involvement. Testing several protein kinase inhibitors: staurosporine (broad spectrum inhibitor), calphostin C (PKC), H-89 (PKA), and PKI-(14—22) (PKA), demonstrated that the PKA inhibitors reduced the level of trafficking of ATP7A, although the trafficking could not be further stimulated with forskolin, a PKA activator (42). Thus the nature of the kinase that phosphorylates Cu-ATPases remains enigmatic.

2. Role of Rab GTPases, Cdc4, and kinase-mediated phosphorylation in Cu-ATPase trafficking

Rab proteins are organelle-specific GTPases with important role in protein trafficking (38). Immunostaining experiments illustrate that soon after treatment of cells with copper, ATP7A is colocalized with Rab7, while later it is sustained with Rab5 (202); however, the functional consequences of these colocalizations have not been in-vestigated. Another protein modulator, Cdc42, is known to regulate trafficking of membrane proteins from the TGN to the basolateral membrane (191). Although dominant negative mutant of Cdc42 did not inhibit copper-dependent relocalization of ATP7A in HeLa cells, constitutively active Cdc42 prevented trafficking of ATP7A to the plasma membrane (42). Thus Cdc42 may contribute to ATP7A trafficking to the plasma membrane by altering cytoskeleton properties (42).

The kinase-mediated phosphorylation of Cu-ATPases appears to play an important role in their intracellular localization. Metabolic labeling experiments in hepatic cells demonstrated that ATP7B is phosphorylated by a kinase and the level of phosphorylation increases following cell exposure to elevated copper (284). The phosphorylation of ATP7B is copper specific and reversible, i.e., following decrease in copper concentration ATP7B is de-phosphorylated. Interestingly, the level of phosphorylation correlates with the intracellular location of the copper transporter. A basal level of phosphorylation of ATP7B is observed at the TGN, while in the vesicles ATP7B is hyperphosphorylated (284). ATP7A expressed in CHO-K1 cells also undergoes a kinase-mediated phosphorylation; in both Cu-ATPases, the amino acid that is phosphorylated was shown to be a serine residue by phosphoamino acid analysis (285, 292).

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3. Regulation of Cu-ATPase trafficking by copper chaperone Atox1

The above scenarios of copper-dependent trafficking of Cu-ATPase all include the assumption that it is the ATPase that senses and responds to changes in the intracellular copper concentration. Although it is likely to be the case, other molecules may contribute to the response. Genetic deletion of copper chaperone Atox1 in mice re- sults in a phenotype that is consistent with the poor delivery of copper to ATP7A, and hence diminished ATP7A function. In addition, the time and dose dependence of ATP7B trafficking were both higher in the Atox1−/− fibroblasts (87). This result supports the model in which copper transport by ATP7A into the lumen of the
secretory compartment rather than high intracellular copper are necessary to initiate ATP7A exit from the TGN. It is also interesting that although the trafficking function of ATP7A in cells lacking Atox1 is altered, it is not abolished, pointing to the alternative/compensatory modes of copper delivery to ATP7A.

In vitro, the holo-Atox1 transfers copper to the NH$_2$-terminal domain of ATP7B, while apo-Atox1 can remove copper from sites in the NH$_2$-terminal domain and thus reset the transporter (300). It is tempting to speculate that in elevated intracellular copper, the regulatory sites in the NH$_2$-terminal domain of Cu-ATPases are saturated with copper and that in this state the Cu-ATPase is directed towards vesicles (where it can be retained through kinase-mediated phosphorylation, see above). When the levels of copper decrease, the apo-Atox1 would remove copper from the regulatory sites in the NH$_2$-terminal domain and generate the form of Cu-ATPase, which is suitable for the targeting to the TGN (Fig. 7).

4. Murr1 (COMMD1) and dynactin subunit p62 interact specifically with ATP7B

Bedlington terriers are purebred dogs that have a high incidence of copper toxicosis due to a defect in biliary copper excretion. These dogs do not have mutations in ATP7B but display a phenotype consistent with inactivation of copper export by ATP7B. The affected gene, COMMD1 (formerly MURR1), encodes a 24-kDa protein with no apparent similarity to other proteins with known function (286). Immunocytochemistry experiments revealed that COMMD1 has a vesicular staining pattern and partially colocalizes with the transferrin receptor that is known to traffic in the recycling endosomes (125). Therefore, it appears that COMMD1 may play a role in polarized delivery of ATP7B to the apical cell compartment. In vitro, COMMD1 specifically interacts with ATP7B through the NH$_2$-terminal region of the protein (271), while in cells it shows specific interactions with XIAP, a recently discovered modulator of copper metabolism and NFkB activity (32, 188). How regulation of NFkB intersects with copper homeostasis and what is the exact role of COMMD1 in a biliary copper export is a subject of great interest.

COMMD1 does not coimmunoprecipitate with ATP7A (271) and could be a specific regulator of hepatic ATP7B. Another protein specifically interacting with ATP7B and likely to be involved in its intracellular trafficking is dynactin 4 (156). The dynactin subunit p62 was initially identified as an interacting partner of ATP7B using yeast two-hybrid assay, and the relevance of this interaction was confirmed by coimmunoprecipitation of both proteins from mammalian cells (156). It is interesting that ATP7B/p62 interactions are facilitated by copper and that the NH$_2$-terminal segment between MBD4 and MBD6 of ATP7B is involved in the interaction (156). These data are consistent with the model discussed in the earlier sections of this review, where copper binding to the NH$_2$-terminal domain of ATP7B induces conformational changes that disrupt interdomain interactions within ATP7B and open sites for interaction between the Cu-ATPase and the components of protein trafficking machinery. Further functional studies of interacting partners of Cu-ATPases are bound to yield new insights into molecular mechanism of regulating localization of Cu-ATPases in a cell.

VII. CONCLUSION

In conclusion, Cu-ATPases are fascinating molecular machines, in which transport activity is required for normal function of many organs in human body. The activity of Cu-ATPases is tightly regulated through complex interactions with modulatory proteins and in response to signaling pathways. Studies on Cu-ATPase structure, mechanism, and regulation are rapidly progressing, and new discoveries keep one intrigued and captivated by sophistication and complexity of copper transport in the human body.

NOTE ADDED IN PROOF

An important work by Nyasae et al. (196) has been published since this manuscript was accepted for publication. In this study, the authors carefully examined the localization and trafficking of endogenous ATP7A in polarized epithelia under physiologically relevant copper concentrations and found that the vast majority of endogenous ATP7A is localized intracellularly under all copper conditions (196). This observation strongly supports the idea of vesicle-mediated copper efflux rather than direct transporter-mediated export of copper across the plasma membrane.

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