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Topical Review

Function and Regulation of the Mammalian Copper-transporting ATPases: Insights from Biochemical and Cell Biological Approaches

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Abstract. Copper is an essential trace element that plays a very important role in cell physiology. In humans, disruption of normal copper homeostasis leads to severe disorders, such as Menkes disease and Wilson's disease. Recent genetic, cell biological, and biochemical studies have begun to dissect the molecular mechanisms involved in transmembrane transport and intracellular distribution of copper in mammalian cells. In this review, we summarize the advances that have been made in understanding of structure, function, and regulation of the key human copper transporters, the Menkes disease and Wilson's disease proteins.

Key words: Copper — Menkes disease — Wilson's disease — ATP7A — ATP7B — Transport — Trafficking — Regulation

Introduction

In the past several years, the studies of metal metabolism, and particularly copper transport and its regulation have undergone remarkable growth and expansion. The impetus for these studies has been provided by the discovery of human copper-transporting ATPases MNKP (or ATP7A) and WNDP (or ATP7B), the first proteins shown to be actively involved in balancing copper concentration in a cell. Copper is an essential nutrient for all organisms because it is required by a variety of enzymes that are involved in critical areas of metabolism. In mammals, these enzymes include cytochrome c oxidase, the terminal enzyme in the electron transport chain; lysyl oxidase, essential for the crosslinking of collagen and elastin; dopamine-β-hydroxylase, required for the synthesis of catecholamines; Cu/Zn superoxide dismutase (SOD), involved in antioxidant defense; tyrosinase, involved in pigmentation, and others. However, copper is also potentially toxic when allowed to accumulate beyond cellular needs. The genetic disorders that affect copper homeostasis illustrate harmful consequences of either copper deficiency or copper accumulation. In humans, the best-known disorders of copper metabolism are Menkes disease and Wilson's disease. Identification of genes *ATP7A* and *ATP7B*, mutated in patients with Menkes and Wilson's diseases, respectively, led to the discovery of human copper-transporting ATPases and opened a new era in understanding of molecular mechanisms that control distribution of copper in living cells.

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Menkes Disease and Wilson's Disease: Clinical Aspects

Menkes disease is an X-linked recessive disorder of copper deficiency with a reported incidence of 1:100,000 births (Danks, 1995). A primary defect in Menkes disease is the reduced transport of dietary copper across the basolateral membrane of enterocytes to hepatic portal circulation. This results in the entrapment of copper within the intestinal mucosa, ultimately leading to a severe copper deficiency in peripheral organs (Danks et al., 1972). Copper transport across the blood-brain barrier is also impaired, and this additional blockage in copper transport compounds the copper deficiency in the central nervous system. Affected Menkes infants are born copper-deficient due to impaired placental copper transport to the developing fetus, and exhibit many of the symptoms associated with severe copper

deficiency. These include neurological impairment (poor head control and visual tracking), convulsions, skeletal abnormalities (wormian bones and rib fractures), tortuosity of cerebral blood vessels, skin laxity and hypopigmentation (Mann, Camakaris & Danks, 1980; Kaler, 1998). Classical Menkes disease is usually lethal by the age of three, however, there are two milder allelic variants of the disorder, called occipital horn syndrome (OHS) and mild Menkes disease. All three forms of Menkes diseases are caused by a similar abnormality in copper metabolism, however, each differs in clinical presentation and survival potential. OHS patients have a prominent protuberance of the occipital bone that is absent in classical Menkes patients. Additional OHS symptoms include skeletal abnormalities (osteoporosis) and connective tissue defects (laxity of the skin and joints and tortuous blood vessels) (Byers et al., 1980; Kuinaviemi et al., 1982; Proud et al., 1996). OHS patients have borderline average intelligence and can survive to adulthood. Mild Menkes disease is extremely rare and is characterized by neurodevelopmental delays and mild ataxia (Procopis, Camakaris & Danks, 1981; Culotta and Gitlin, 2001).

Wilson's disease is an autosomal recessive disorder of copper metabolism with a reported incidence of 1:30,000 births (Danks, 1995). Wilson's disease is characterized by the progressive intracellular accumulation of copper in the liver and several other tissues (Scheinberg & Sternlieb, 1984; Harris & Gitlin, 1996; Culotta and Gitlin, 2001).

Elevated hepatic copper in Wilson's disease patients causes liver pathology, which can be manifested as acute or chronic hepatitis, cirrhosis, and fulminant hepatic failure (Riordan & Williams, 2001). Copper can also rise to toxic levels in the brain, leading to neurological and psychiatric abnormalities, which include diminution in facial expressions, tremors, dystonia, abnormal behavior, personality changes, and depression (Loudianos & Gitlin, 2000). Most Wilson's disease patients have reduced copper incorporation into ceruloplasmin, which is the major copper-containing protein of serum (Scheinberg & Sternlieb, 1984).

Impaired Copper Efflux in Cells and Tissues of the Menkes Disease and Wilson's Disease Patients

One of the primary defects in both Menkes disease and Wilson's disease is the markedly reduced efflux of copper from cells. Cultured skin fibroblasts, amniocytes, and lymphoblasts from Menkes patients accumulate high levels of copper when allowed to grow in essential medium without additional copper for periods of 3–5 days (Goka et al., 1976; Horn, 1976). Kinetic studies with the ^{64}Cu radioisotope have demonstrated that reduced copper efflux accounts for the copper accumulation in Menkes patient cells (Camakaris et al., 1980). Surprisingly, cells from Menkes patients with milder forms of the disease accumulate copper to the same extent as those from classical Menkes patients (Peltonen et al., 1983). Similarly, primary cell cultures derived from different mottled mice also accumulate copper to the same extent, even though these animal models of Menkes disease display a broad phenotypic diversity (Masson et al., 1997). Hence, although a disease diagnosis can be made from studying cultured cells, neither the severity of the disease nor the molecular basis of the clinical variation has been predicted from studies of copper efflux.

In Wilson's disease, the hepatic copper content is elevated, while the concentration of copper in the bile, a major excretion route for copper, is markedly decreased. Similarly, Long-Evan Cinnamon rats, which have a deletion in the gene homologous to the Wilson's disease gene (Wu et al., 1994), are characterized by excessive copper accumulation in the liver and impaired biliary excretion of copper (Suzuki & Aoki, 1994; Sugawara et al., 1995; Schilsky et al., 2000). Thus, both Menkes disease and Wilson's disease appear to stem from a similar underlying defect in copper efflux. This assertion is supported by studies that demonstrate complementation of the defective copper efflux in Menkes fibroblasts with the expression of the recombinant Wilson's disease protein (La Fontaine et al., 1998; Payne & Gitlin, 1998).

How does a similar defect in copper efflux give rise to two very different diseases of copper metabolism? The clinical difference between Menkes and Wilson's patients is probably due to the difference in tissue-specific expression of the respective proteins. Menkes disease mRNA is expressed in the enterocytes and the endothelium of the blood brain barrier as well as in a number of other tissues (Vulpe et al., 1993; Paynter et al., 1994; Murata et al., 1997). Accordingly, in Menkes patients a defect in copper efflux via Menkes disease protein reduces dietary copper absorption and the copper entry to the central nervous system, respectively. This leads to systemic copper deficiency. In contrast, Wilson's disease protein is expressed predominantly in the liver and, at lower levels, in the brain, kidneys, placenta, and the heart (Tanzi et al., 1993; Vulpe et al., 1993; Paynter et al., 1994; Kuo, Gitschier & Packman, 1997; Murata et al., 1997; Saito et al., 1999). Defects in the function of this protein disrupt biliary excretion of copper, causing a toxic buildup of copper in the liver. Increased levels of copper were also detected in other tissues expressing WNDP (Scheinberg & Sternlieb, 1984).

Menkes Disease and Wilson's Disease Proteins: Structural Features

The genes affected in patients with Menkes disease and Wilson's disease were identified in 1993–94 (Bull

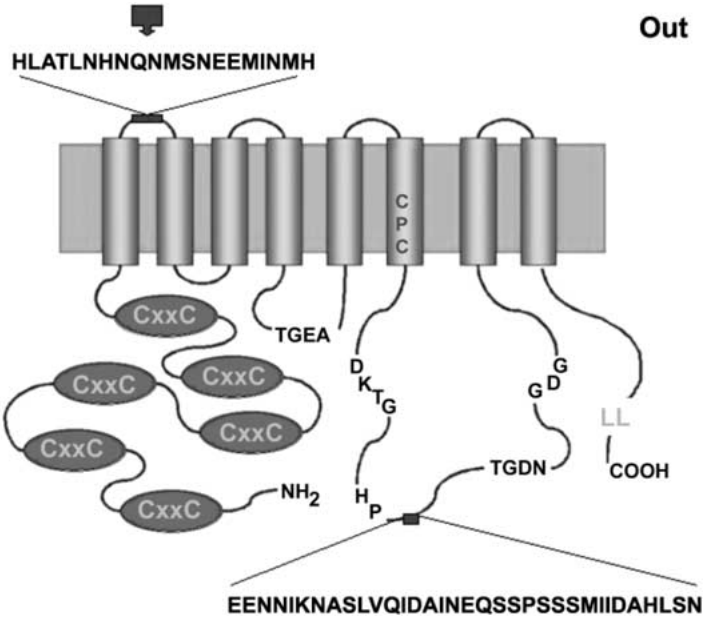


Fig. 1. The predicted transmembrane organization of the Menkes disease protein (MNKP) and Wilson's disease protein (WNDP), the human copper-transporting ATPases ATP7A and ATP7B. The ovals with the CxxC sequence in the N-terminal domain (NTD) indicate the metal-binding repeats (MBR) containing the conserved copper-binding motif GMTCxxCxxxIE; "C" is a Cys residue directly involved in copper coordination. The letters LL at the C-terminal region of the protein mark the location of the di-leucine motif. DKTG, TGEA, TGDN, and AMX**GD**GNVD (indicated by the letters GDG) are the sequence motifs characteristic for all P-type ATPases; CPC and HP sequences are conserved in the P-type ATPases involved in transport of transition metals. The blue rectangles and corresponding sequences indicate the sequence inserts in MNKP, the arrow shows the position of the glycosylation site.

et al., 1993; Chelly et al., 1993; Mercer et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993; Yamaguchi, Heiny & Gitlin, 1993; Petrukhin et al., 1994) and were named *ATP7A* and *ATP7B*. Analysis of the corresponding cDNA sequences revealed that Menkes disease and Wilson's disease proteins (MNKP and WNDP, respectively) are large membrane proteins (165–180 kDa) with predicted 8-transmembrane-segments topology (Fig. 1). The proteins are 54% identical over their entire sequence, although their N-terminal portions are less similar than their C-terminal parts. This uneven distribution of homology along the protein sequence of MNKP and WNDP is likely to reflect the division of labor between the N-terminal and C-terminal portions of these proteins: the C-terminal half is essential for catalytic and transport activity of MNKP and WNDP, while the N-terminal region is likely to play an important role in their regulation (*see* below). MNKP is glycosylated (Yamaguchi et al., 1996), and is about 30–40 amino-acid residues longer than WNDP due to the presence of two sequence inserts (Fig. 1). The first insert is located in the extracellular loop and carries a consensus glycosylation site. The amino-acid sequence of this insert is His/Met-rich, suggesting its possible role in the copper transport step. The second sequence insert is in the central cytosolic loop.

MNKP and WNDP belong to a large family of cation-transporting P-type ATPases, a group of integral membrane proteins that translocate ions across cell membranes, using the energy of ATP hydrolysis (Lutsenko & Kaplan, 1995; Scarborough, 1999). The family consists of more than 200 members with representatives in all living organisms, including bacteria, yeast, plants, and mammals (Palmgren & Axelsen, 1998). MNKP and WNDP were the first P-type ATPases shown to be involved in the transport of copper. Following the discovery of MNKP and WNDP, copper-transporting ATPases homologous to these human transporters were found in a wide range of species from bacteria to mammals (The classification and protein-sequence alignments for all members of this family can be found at the "P-type ATPases" web site at http://biobase.dk/~axe/Patbase.html).

Like all P-type ATPases, MNKP and WNDP have several characteristic sequence motifs, TGES/A, DKTG, TGDN, AMXGDGNVD, located in two cytosolic loops (Fig. 1). In a number of P-type ATPases, these signature motifs were shown to be essential for ATP binding and hydrolysis as well as for conformation changes that accompany these processes (for example, Post & Kume, 1973; Clarke, Loo & MacLennan, 1990; McIntosh et al., 1999; Clausen et al., 2001; Jorgensen, Jorgensen & Pedersen, 2001). Several mutations causing Wilson's disease were shown to lie within or very close to the signature sequences, consistent with the important role of these regions for the functional activity of WNDP. For example, the T1031S and T1033A substitutions were found in the DKTGT^{1031}IT^{1033} segment, T1220M and D1222Y mutations–in the TGDN motif, the G1266R,Y and D1267A mutations were detected in the AMVG^{1266}D^{1267} GNVD sequence, etc. (The complete list of the Wilson's disease mutations can be found at http://www.medgen.med.ualberta.ca/database.html).

Along with the sequence motifs that are present in all P-type ATPases, MNKP and WNDP have characteristic structural features, which are related to their specific function of transporting copper. These features include the CPC motif, commonly found in

the P-type ATPases that transport transition metals (the subfamily of the P_1-type or CPX ATPases; Lutsenko & Kaplan, 1995; Solioz & Vulpe, 1996). At their N-terminal portion, both MNKP and WNDP contain six repetitive sequences, each carrying the metal-binding motif GMT/HCxxCxxxIE (Fig. 1).

Biochemical Properties of the Human Copper-transporting ATPases

The presence of the P-type ATPase signature motifs in the primary structure of both MNKP and WNDP provided the first hint that copper transport by these proteins requires ATP hydrolysis. It also suggested that the basic molecular mechanism of copper transport was likely to resemble the ATP-driven transport of other ions, such as calcium or sodium. For these reasons, the initial efforts in characterization of the functional properties of MNKP and WNDP were focused on testing whether these two proteins function as P-type ATPases.

Experiments utilizing vesicular preparations confirmed that transport of copper by either MNKP or WNDP requires ATP (Voskoboinik et al., 1998, 2001a). To investigate the catalytic mechanism of MNKP and WNDP in more detail, two approaches have been utilized. MNKP was overexpressed in CHO-K1 cells and the catalytic properties of this protein were studied following immunoprecipitation (Voskoboinik et al., 2001b). WNDP was expressed in insect cells using baculovirus-mediated infection; the high levels of protein expression allowed omission of the immunoprecipitation step and permitted characterization of the expressed WNDP in membrane preparations of Sf9 cells (Tsivkovskii et al., 2002).

Typically, hydrolysis of ATP by P-type ATPases is accompanied by the transfer of γ-phosphate of ATP to the invariant Asp residue in the DKTG motif (Fig. 1) and by formation of a transient acyl-phosphate intermediate; the reaction is stimulated by the exported ion. In agreement with this prediction, the incubation of MNKP or WNDP with ATP results in formation of the phosphorylated product with properties typical for an acyl-phosphate intermediate. The phosphorylation is transient, i.e., the incorporation of radioactive phosphate can be decreased by subsequent addition of cold ATP or reversed by addition of ADP (Voskoboinik et al., 2001b; Tsivkovskii et al., 2002). The phosphorylated intermediate is also sensitive to treatment with hydroxylamine or high pH, and the mutation of the invariant Asp residue in the DKTG sequence motif to Ala or Glu abolishes this phosphorylation (Voskoboinik et al., 2001b; Tsivkovskii et al., 2002). Overall, MNKP and WNDP behave very similarly with respect to this key enzymatic step, although the rate of MNKP phosphorylation appears to be faster than the rate of WNDP phosphorylation.

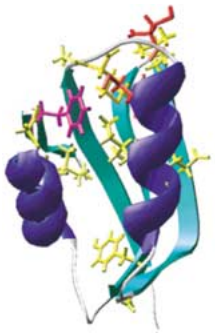
These initial studies provided strong evidence that both MNKP and WNDP function as copper-transporting P-type ATPases. However, many important questions remain. The number of copper atoms transported per molecule of hydrolyzed ATP is still unknown. It is also unclear whether copper transport by MNKP and WNDP is electrogenic and whether or not a counter ion is involved. Similarly, it remains to be determined how copper is released from the transporters after the ion traverses the lipid bilayer, i.e., whether the change in conformation of the protein is sufficient to decrease the affinity of enzyme for copper and stimulate the release, or copper is retrieved from the transporters by specific acceptor proteins or by low molecular weight compounds.

The Role of the N-Terminal Domain in Functional Activity and Regulation of Copper-transporting ATPases

The N-terminal domain (NTD) of MNKP and WNDP represents a unique structural feature of human copper-transporting ATPases. The domain contains six metal-binding repeats (MBR), which are about 70 amino-acid residues long, and are 20–60% homologous (Figs. 1 and 2). The fairly low similarity between some MBRs suggests that they may have distinct properties, possibly with respect to protein interactions with the copper chaperone Atox1, which serves as an intracellular copper donor for MNKP and WNDP (Hamza et al., 1999, 2001), or with other proteins.

NMR studies of the fourth MBR of MNKP (Gitschier et al., 1998) revealed that the MBRs represent a compact structural unit with the ferredoxin-like fold (Fig. 2). Within each MBR, the highly conserved motif, GMTC/HxxCxxxIE, forms a loop that houses the copper-binding site CxxC. Interestingly, the residues conserved in all metal-binding repeats are clustered close to this loop (Fig. 2), suggesting that these residues may be important in providing the appropriate environment necessary to accept copper from intracellular metal donors.

Studies from several laboratories demonstrated that the NTD of both MNKP and WNDP bind copper readily *in vitro* and in a cell with the total stoichiometry of approximately 6 copper atoms per domain (DiDonato et al., 1997; Lutsenko et al., 1997; Jensen et al., 1999b). These results suggested that each metal-binding repeat in the NTD is involved in copper coordination, and recently the ability of a single MBR to coordinate copper was directly demonstrated (Harrison, Meier & Dameron, 1999; Jensen et al., 1999a; Cobine et al., 2000). X-ray absorption spec-



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MBR1-MNKP  VNSVTISVEG MTCNSCVWTI EQQIGKVNGV HHIKVSLEEK NATIIYDPKL QTPKTLQEAI DDMGFDAVIH
MBR1-WNDP  VATSTVRILG MTCQSCVKSI EDRISNLKGI ISMKVSLEQD SATVKYVPSV VCLQQVCHQI GDMGFEASIA
MBR2-MNKP  EVVLKMKVEG MTCHSCTSTI EGKIGKLQGV QRIKVSLDNQ EATIVYQPHL ISVEEMKKQI EAMGFPAFVK
MBR2-WNDP  EAVVKLRVEG MTCQSCVSSI EGKVRKLQGV VRVKVSLSNQ EAVITYQPYL IQPEDLRDHV NDMGFEAAKS
MBR3-MNKP  DSTATFIIDG MHCKSCVSNI ESTLSALQYV SSIVVSLENR SAIVKYNASS VTPESLRKAI EAVSPGLYRV
MBR3-WNDP  VVTLQLRIDG MHCKSCVLNI EENIGQLLGV QSIQVSLENK TAQVKYDPSC TSPVALQRAI EALPPGNFKV
MBR4-MNKP  TQETVINIDG MTCNSCVQSI EGVISKKPGV KSIRVSLANS NGTVEYDPLL TSPETLRGAI EDMGFDATLS
MBR4-WNDP  CSTTLIAIAG MTCASCVHSI EGMISQLEGV QQISVSLAEG TATVLYNPSV ISPEELRAAI EDMGFEASVV
MBR5-MNKP  SSKCYIQVTG MTCASCVANI ERNLRREEGI YSILVALMAG KAEVRYNPAV IQPPMIAEFI RELGFGATVI
MBR5-WNDP  PQKCFLQIKG MTCASCVSNI ERNLQKEAGV LSVLVALMAG KAEIKYDPEV IQPLEIAQFI QDLGFEAAVM
MBR6-MNKP  DGVLELVVRG MTCASCVHKI ESSLTKHRGI LYCSVALATN KAHIKYDPEI IGPRDIIHTI ESLGFEASLV
MBR6-WNDP  DGNIELTITG MTCASCVHNI ESKLTRTNGI TYASVALATS KALVKFDPEI IGPRDIIKII EEIGFHASLA
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Fig. 2. The structure of the isolated MBR (*left*) and the sequence alignment of the metal-binding repeats (MBRs) in the N-terminal domains of MNKP and WNDP (*right*). The figure was generated using the published NMR solution structure for the MBR4 of MNKP (Protein Data Bank accession number 1AWO, Gitschier et al, 1998). The cysteine residues involved in copper coordination are shown in red, the residues conserved in all 6 metal-binding repeats of both MNKP and WNDP are yellow. Notice that the highly conserved Phe (purple) is missing in the MBR3 of both MNKP and WNDP; in this, MBR the conserved Thr residue in the GMTCxxC loop is replaced with His.

troscopy experiments revealed that copper binds to MBR in the reduced Cu(I) form, and that two cysteines are the major ligands coordinating copper (Ralle et al., 1998; DiDonato et al., 2000). Binding of copper to Cys residues in the metal-binding sites protects the NTD against labeling with the Cys-directed fluorescent coumarine maleimide CPM; this property can be utilized as a convenient test to assay copper occupancy of isolated NTD (Lutsenko et al., 1997). It has been also shown that binding of copper to NTD induces changes in its secondary structure (DiDonato et al., 2000). These copper-dependent conformational changes in NTD are likely to play a very important role in the multiple regulatory effects that copper exerts on MNKP and WNDP (*see* below).

Recent studies have provided convincing evidence that the presence of all six copper-binding sites is not necessary for catalytic activity of MNKP and WNDP. Also, it has become clear that the copper-binding sites in NTD are functionally non-equivalent, i.e., the sites that are closest to the membrane portion cannot be substituted with the other N-terminal metal-binding sites without loss of the copper-transporting activity of the ATPase (Forbes et al., 1999). Interestingly, it remains uncertain whether at least one copper-binding repeat must be present for the ATP-driven transport to occur. The experiments with various NTD mutants of MNKP and WNDP expressed in mammalian cells suggest that at least one site is necessary for transport activity of MNKP and WNDP (Iida et al., 1998; Payne & Gitlin, 1998; Forbes et al., 1999). However, recent experiments by Voskoboinik et al. (2001b) have provided evidence that the N-terminal copper binding sites are not essential for catalysis per se, but rather serve in copper acquisition when copper concentrations are low.

These authors demonstrated that the MNKP mutant, in which all Cys residues in the NTD were substituted with Ser, was able to undergo copper-dependent catalytic phosphorylation (Voskoboinik et al., 2001b). Hence, when copper is not limiting, it can bypass the NTD and stimulate catalytic phosphorylation, possibly through direct binding to other ligands such as the intramembrane CPC motif (Fig. 1). Significantly, the NTD mutant protein appeared to have lower affinity for copper and had no detect-

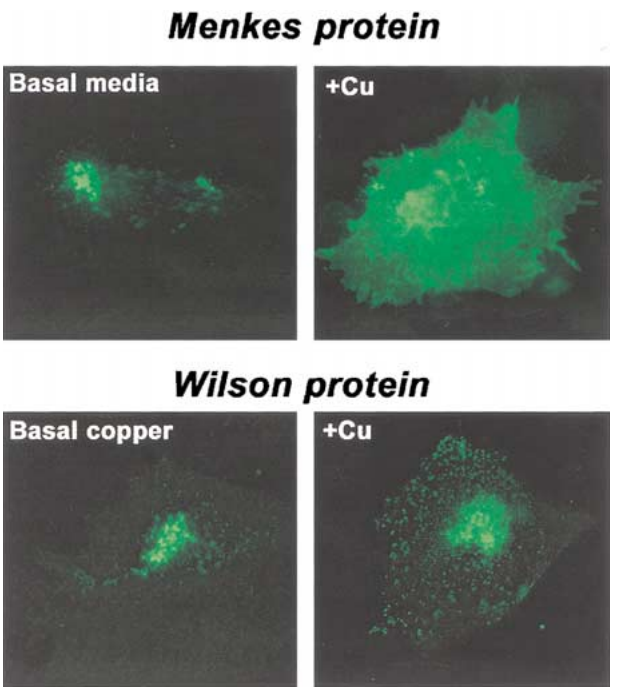

Fig. 5. Copper-dependent localization of the MNKP and WNDP. Immunostaining with specific antibody and fluorescence microscopy demonstrates typical perinuclear localization of MNKP and WNDP in the trans-Golgi network of CHO cells cultured in basal minimal media (*left photos*). Under elevated copper conditions, 2 hr 100 μM $CuCl_2$ (+*Cu*), both proteins traffic to post-TGN compartments. MNKP relocalizes to the plasma membrane (*top right*), whereas WNDP is recruited to the vesicles (*bottom right*).

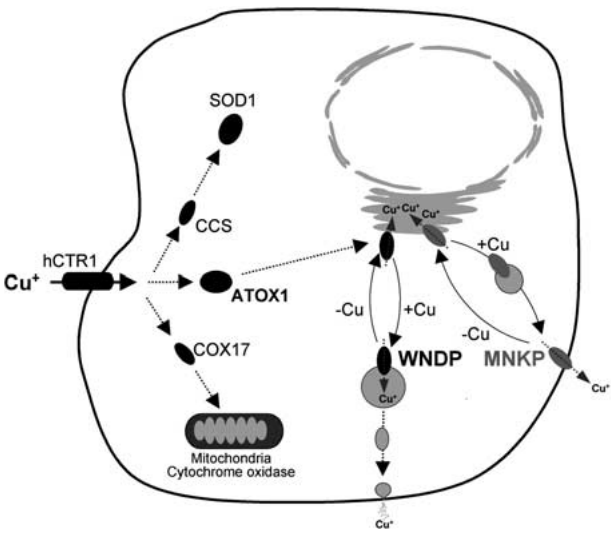

Fig. 3. Schematic illustration depicting copper distribution in a generalized human cell. Copper uptake via hCTR1 provides copper to the copper chaperones ATOX1, CCS and COX17 for the transfer of copper to MNKP/WNDP, Cu/Zn superoxide dismutase (SOD) and cytochrome c oxidase. MNKP and WNDP are shown located in the TGN, and the copper-induced recycling pathways are denoted by arrows. MNKP and WNDP in the TGN provide copper to secreted cuproenzymes, such as tyrosinase and ceruloplasmin. Under excessive copper conditions, MNKP and WNDP are sorted to post-TGN vesicles for exocytic trafficking. WNDP traffics to a cytoplasmic vesicle compartment where it sequesters excess copper for subsequent excretion via a mechanism that presumably does not require WNDP to be located at the plasma membrane. Elevated copper induces the trafficking of MNKP to the plasma membrane of cells. As cytosolic copper concentrations decrease, MNKP and WNDP resume a steady-state TGN location.

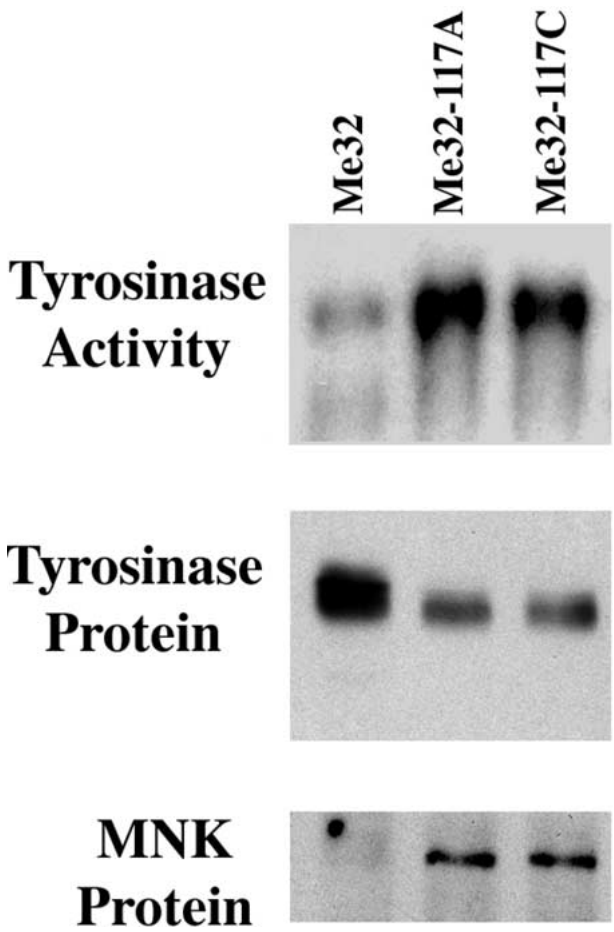

Fig. 4. MNKP-dependent activation of tyrosinase determined by SDS-PAGE. Transfection of a tyrosinase expression plasmid into the Menkes patient cell line, Me32, results in a low level of tyrosinase activity. Coexpression of both MNKP and tyrosinase proteins in Me32 cells to create the Me32-117A and Me32-117B cell lines, results in tyrosinase activation. Tyrosinase activity is measured colorimetrically in non-reducing SDS-PAGE gels incubated in the tyrosinase substrate, L-DOPA.

able ^{64}Cu-translocating activity in the regular assay medium (Voskoboinik et al., 2001b). These findings suggest that the role of the NTD is to enable high-affinity copper binding under low intracellular copper conditions, presumably via exchange with the copper chaperone ATOX1.

It is also possible that the NTD is involved in regulation of catalytic activity of human copper-transporting ATPases by interacting with other domains of the copper ATPases and facilitating conformational transitions during the catalytic cycle. Such NTD-dependent conformational transitions may control the affinity of the intramembrane binding sites for copper. Consistent with this hypothesis, the ability of WNDP to become catalytically phosphorylated in response to copper depends on whether or not WNDP has some copper pre-bound, presumably to its NTD (Tsivkovskii et al., 2002). WNDP, expressed in Sf9 cells, has a high affinity for copper and is active even when the concentration of copper in the buffers is low. Treatment with copper chelator, which apparently strips copper from the NTD, converts WNDP into a state with lower apparent affinity for copper, and in this case, higher concentrations of copper are needed to stimulate the WNDP activity (Tsivkovskii et al., 2002). Also, in agreement with the possible role of the NTD in copper-dependent conformational changes, the MNKP mutant lacking the metal-binding cysteines in the NTD appears to be stabilized in a conformation with lower affinity for inorganic phosphate, P_i, a product of the catalytic reaction (Voskoboinik et al., 2001b).

MNKP and WNDP Transport Copper to Cuproenzymes in Secretory Compartments

MNKP and WNDP are located in the final compartment of the Golgi complex, known as the trans-Golgi network (TGN) (Dierick et al., 1997; Hung et al., 1997; Petris et al., 1996; Yamaguchi et al., 1996). It is believed that the physiological function of both proteins in the TGN is to deliver copper into the

secretory pathway to copper-dependent enzymes (Fig. 3). In the yeast *Saccharomyces cerevisiae*, the MNKP/WNDP homologue, Ccc2p, is located in the late-Golgi compartment and transports copper into the lumen of the secretory compartment to Fet3p, a protein that is involved in iron uptake. Deletion of ccc2 disrupts the copper-dependent ferroxidase activity of Fet3, resulting in diminished iron uptake and growth retardation under iron-limiting conditions (Yuan et al., 1995). Both MNKP and WNDP can complement ccc2 mutants by allowing growth under iron-limiting conditions (Hung et al., 1997; Payne, Kelly & Gitlin, 1998). These observations suggested that the MNKP and WNDP transport copper to secreted cuproenzymes in the TGN in mammalian cells.

Recent experiments in mammalian cells and tissues have supported this assertion. In LEC rats, the Wilson disease animal model, incorporation of copper into apo-ceruloplasmin is restored after infusion of a recombinant adenovirus bearing the WNDP cDNA (Terada et al., 1998). More recent experiments have shown reduced activity of a recombinant form of the copper-dependent enzyme, tyrosinase, in cultured Menkes-patient fibroblasts. However, when MNKP was coexpressed in these cells, tyrosinase was activated (Petris, Strausak & Mercer, 2000) (Fig. 4). These findings support a dual role for MNKP and WNDP in the efflux of excessive cytoplasmic copper, and the metallation of secreted cuproenzymes. Studies investigating the subcellular location of MNKP and WNDP have provided valuable insights into how MNKP and WNDP are distributed to the appropriate intracellular compartments to perform both these copper transport functions.

The Copper-dependent Localization of MNKP and WNDP

The subcellular distribution of MNKP and WNDP in cultured cell lines was investigated using immunocytochemistry (Petris et al., 1996; Yamaguchi et al., 1996; Dierick et al., 1997; La Fontaine et al., 1999). In human and rodent cell lines, MNKP was shown to localize to the TGN, and this localization has also been confirmed in vivo, using sections of human breast tissue (Ackland et al., 1999). Similar studies have also shown that WNDP is located in the TGN of hepatoma cell lines, primary hepatocytes and in rat and human liver sections (Hung et al., 1997; Nagano et al., 1998; Schaefer & Gitlin, 1999, Schaefer et al., 1999; Roelofsen et al., 2000). As discussed in the previous section, the location of MNKP and WNDP in the TGN presumably enables the transport of cytoplasmic copper to cuproenzymes that are synthesized in secretory compartments.

The TGN is a "sorting stations" where proteins are differentially sorted into vesicles to be distributed to various destinations in the cell. A novel post-translational mechanism for regulating copper export via MNKP and WNDP has been identified, involving the copper-dependent exocytic trafficking of these proteins from the TGN (Figs. 3 and 5) (Hung et al., 1997; Petris et al., 1996). The trafficking of both copper ATPases does not involve *de novo* protein synthesis, and occurs at media copper levels in the 20–40 μM range. In the case of MNKP, elevated copper induces the relocation from the TGN to the plasma membrane (Fig. 3) (Petris et al., 1996). Unlike MNKP, the WNDP is not recruited to the plasma membrane. In cultured hepatoma cell lines, elevated copper induces the trafficking of WNDP to cytoplasmic vesicles (Hung et al., 1997), and apical vacuoles reminiscent of bile canaliculi (Roelofsen et al., 2000). WNDP also trafficks to a vesicular compartment in non-hepatic cell lines such as CHO cells (Forbes & Cox, 2000; La Fontaine et al., 2001). These compartments are devoid of marker proteins for late endosomes and lysosomes and may represent a novel compartment for storing excess copper. Copper-induced trafficking of WNDP to vesicles located in proximity to the canalicular membrane has been observed in liver sections derived from rats injected with excess copper (Schaefer & Gitlin, 1999). This finding suggests that the trafficking phenomenon is a *bonafide* regulatory mechanism that exists in vivo.

The copper-induced trafficking of MNKP and WNDP is a reversible process, since both proteins return to the TGN when cells are returned from high copper to basal media. This ability to recycle presumably serves to re-establish copper transport to secreted cuproenzymes once basal copper levels are restored. Interestingly, MNKP constitutively cycles between the TGN and the plasma membrane without the addition of copper (Petris & Mercer, 1999). It is unknown whether this cycling is induced by basal copper levels in the media, or is simply a corrective mechanism for retrieving MNKP that has escaped TGN retention. The restoration of TGN localization of both MNK and WND ATPases following the transfer of cells from high to low copper, suggests the existence of sorting signals for endocytic retrieval to the TGN. In the case of MNKP, an endocytic retrieval signal has been mapped to a di-leucine in the cytoplasmic carboxyl terminal region (Petris et al., 1998; Francis et al., 1999) (Fig. 1). Di-leucine motif is known to function in the endocytosis of proteins via clathrin-coated vesicles (Marks et al., 1996). Mutation of these two leucine residues causes MNKP to accumulate at the plasma membrane due to impaired endocytosis (Petris et al., 1998; Francis et al., 1999; Petris & Mercer, 1999). Interestingly, WNDP has three leucine residues that align with the di-leucine motif of MNKP. This sequence similarity suggests that WNDP may also cycle via the plasma membrane. The current difficulties with detection of

WNDP at the plasma membrane could reflect low amounts of WNDP in this location at any given time (Schaefer et al., 1999).

Mutations Affecting Copper-induced Trafficking of MNKP and WNDP

The trafficking of MNKP and WNDP between the TGN and post-TGN compartments provides a means to balance copper transport to secreted cuproenzymes and copper export from the cytoplasm. However, the mechanism for sensing copper levels and triggering the exocytic trafficking of these ATPases is not well understood. Site-directed mutagenesis of MNKP has shown that the copper-induced relocalization is inhibited by mutation of the Cys residues in the CxxC copper-binding motifs of the NTD. However, there is some discrepancy as to the minimal requirements for this relocalization. One study suggests the 5th or 6th copper-binding site must be intact (Strausak et al., 1999), whereas a second report suggests any one of the six copper-binding sites is sufficient (Goodyer et al., 1999). Both studies used identical mutations to alter the copper-binding sites, so the discrepancy in findings may relate to the different cell types used in these studies to express the MNKP constructs.

As described above, the copper-binding sites in the NTD probably act as sensors of intracellular copper, but it is clear that these motifs are not solely responsible for copper-induced trafficking. Several disease-causing mutations in both MNKP and WNDP that prevent the copper-induced trafficking from the TGN have been identified in cytoplasmic and membrane-spanning regions. MNKP trafficking-mutations include A1364V in the seventh transmembrane domain of the human protein (Ambrosini & Mercer, 1999), and a deletion of two amino acids, AL801, in the first cytoplasmic loop of MNKP from the mottled brindled mouse, a Menkes disease animal model (La Fontaine et al., 1999). WNDP trafficking-mutations include G943S (Forbes & Cox, 2000) in the fifth transmembrane region, mutation of the conserved CPC motif within the sixth transmembrane domain (Forbes & Cox, 2000), and M1356V in the eighth transmembrane region of the murine WNDP (La Fontaine et al., 2001). The fact that these trafficking-defective mutations occur in cytoplasmic and membrane-spanning regions, suggests that the mutations are unlikely to affect signals with distinct roles in copper-induced trafficking per se. Rather, these widely distributed trafficking mutations are consistent with a model where the trafficking of MNKP and WNDP is dependent on copper transport, and these mutations indirectly prevent trafficking by altering protein folding and the ability of the enzymes to undergo conformational transitions coupled with their catalytic activity. The proteins involved in recognition and sorting of MNKP and WNDP into exocytic vesicles have not been identified, and are the subject of ongoing research.

Recent studies have shed some light on the molecular events associated with copper-binding to WNDP. These events are likely to contribute to metal-dependent regulation of the copper-ATPase activity and trafficking. First, the copper-dependent conformational changes upon binding of copper to the NTD of WNDP have been demonstrated (DiDonato et al., 2000). Secondly, it has been shown that binding of copper to the NTD of WNDP alters intradomain interactions in this protein (Tsivkovskii, MacArthur & Lutsenko, 2001). Copurification experiments revealed that in its apo-form, the NTD interacts more tightly with the ATP-binding domain of WNDP than it does when it is fully loaded with copper. Importantly, this domain-domain interaction appears to modulate the conformational state of the ATP-binding domain, as evidenced by changes in its nucleotide affinities. The approximately 5-fold increase in affinity for ATP suggests that the dissociation of the domains leads to structural changes in the ATP-binding domain. These conformational transitions in two major functional domains of WNDP and changes in domain-domain interactions are likely to occur during the copper-ATPase functioning. As a result, certain regions of the protein can become exposed for interactions with the intracellular trafficking machinery and/or with other proteins involved in regulation of the transporters (*see* below). Therefore, mutations that lock the copper-ATPases in certain conformations may not only decrease the enzymatic activity of the transporters, but negatively affect their ability to traffic.

Regulation of WNDP by Kinase/Phosphatase Pathways

Recent studies suggest that in addition to modulating the intracellular localization of the copper-transporting ATPases, copper can also regulate the post-translational modifications of these proteins. Experiments utilizing metabolic labeling of cells with inorganic phosphate have demonstrated phosphorylation of WNDP in cells grown in the regular growth medium (total copper concentration is approximately 1 μM). This intracellular phosphorylation is distinct from the catalytic phosphorylation described above, since it can be observed with a catalytically inactive mutant (our unpublished data), and therefore is likely to be a result of modification by a kinase. Also, unlike the acyl-phosphate intermediate, which is formed during ATP hydrolysis by WNDP, the product of non-catalytic phosphorylation is stable and can be

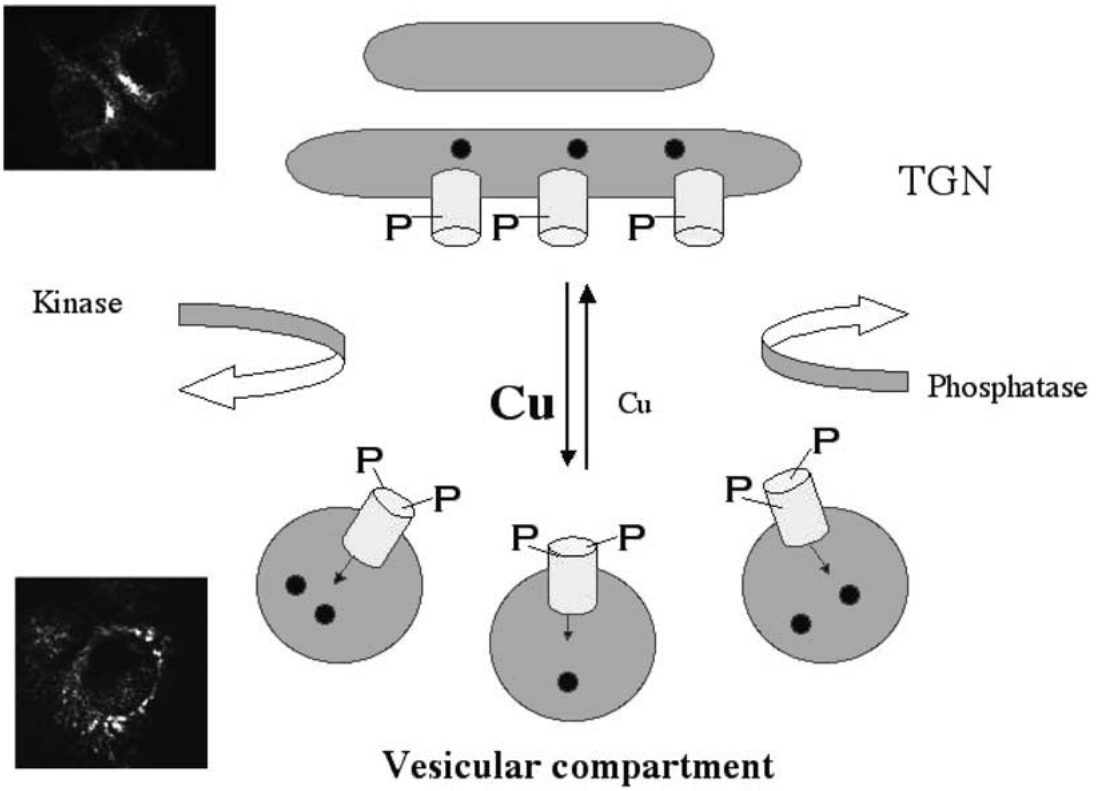

Fig. 6. Copper-dependent phosphorylation and intracellular localization of WNDP in a cell. The cylinders represent WNDP, the letter "P" depicts the phosphate groups added to WNDP by kinase(s). Under standard cell growth conditions, WNDP is localized predominantly in the TGN and has a basal level of phosphorylation. Following copper treatment, WNDP becomes hyperphosphorylated and relocalizes to the vesicular compartment. Removal of copper induces dephosphorylation and return of WNDP to the TGN. The bottom insert illustrates the intracellular localization of the hyperphosphorylated WNDP after treatment of cells with copper and immunostaining with anti-WNDP antibody. The upper insert shows localization of the basally phosphorylated WNDP after excess copper was removed from the growth medium and cells were incubated for 4 hours in the regular medium in the presence of protein synthesis inhibitor.

observed on standard SDS gels following immunoprecipitation of WNDP (Vanderwerf et al., 2001). It is likely that the putative kinase that phosphorylates WNDP is fairly ubiquitous, because non-catalytic phosphorylation can be observed not only in mammalian cell lines, but also in insect cells expressing recombinant WNDP (Vanderwerf & Lutsenko, 2002). In these insect cells, non-catalytic phosphorylation of WNDP was shown to occur at Ser residue(s).

Current data also suggest that copper regulates this kinase-mediated phosphorylation of WNDP. Increases in the copper concentration of the medium results in a 2- to 3-fold stimulation of the kinase-dependent phosphorylation. Return of cells into standard medium leads to the eventual dephosphorylation of the transporter. Experiments using λ-phosphatase demonstrate that the basal phosphorylation and copper-induced phosphorylation are likely to occur at different regions of WNDP (Vanderwerf et al., 2001). The site of basal phosphorylation was mapped to the central region of the protein, and the N-terminal domain or C-terminal tail were shown not to be required for this basal phosphorylation. In contrast, copper-dependent phosphorylation does not occur in the WNDP variants that either lack the N-terminal domain, or have mutations in this region (Vanderwerf et al., 2001). Hence, proper folding or function of the N-terminal domain appears to be important for the copper-dependent phosphorylation by a kinase.

The physiological role of basal and copper-dependent phosphorylation of WNDP is currently unknown. However, it is notable that there is an association between the level of WNDP phosphorylation and its intracellular localization. As shown in Fig. 6, under basal conditions, when WNDP has a basal level of phosphorylation, it has perinuclear TGN-like localization. Addition of copper to the medium stimulates kinase-dependent phosphorylation of WNDP and this is associated with the redistribution of WNDP to the vesicular compartment. Substitution of the copper-containing medium with basal medium leads to dephosphorylation of WNDP, and the return of WNDP back to the TGN (Vanderwerf et al., 2001). These findings suggest that

phosphorylation and trafficking may be coupled, i.e., the copper-regulated phosphorylation may serve as a sorting signal for the trafficking of WNDP. Further experiments will need to address whether kinase-mediated phosphorylation is important to induce the trafficking of the transporter or possibly to retain the transporter in a specific cell compartment. It is also possible that copper-induced phosphorylation regulates the transport activity of WNDP, facilitating sequestration of excess copper into the vesicles.

Conclusions

The human copper-transporting ATPases are likely to represent the primary checkpoints for copper homeostasis at the whole-body level. MNKP and WNDP are complex membrane proteins with dual roles in copper efflux and copper transport to secreted cuproenzymes. Adding to this complexity is the copper-responsive trafficking mechanism, which functions to distribute MNKP and WNDP at appropriate levels between the TGN or post-TGN compartments where these proteins can fulfill their roles. Changes in intracellular copper have multiple effects on MNKP and WNDP, including regulation of their conformational states, catalytic activity, intracellular localization, and the level of kinase-dependent phosphorylation. Indeed, the relative impact of various mutations on each of these functions of MNK and WND ATPases probably accounts for the clinical diversity of Menkes and Wilson's diseases. It is clear that we are only just beginning to understand function and regulation of mammalian copper-transporting ATPases, and further studies are required to elucidate the sophisticated mechanism(s) by which these ATPases control cellular copper distribution. Ultimately, these studies will need to move to transgenic animal models to understand the importance of these functions in the context of organ function and whole body copper homeostasis.

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