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Copper-induced trafficking of the Cu-ATPases: A key mechanism for copper homeostasis

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Abstract

The Menkes protein (MNK) and Wilson protein (WND) are transmembrane, CPX-type Cu-ATPases with six metal binding sites (MBSs) in the N-terminal region containing the motif GMXCXXC. In cells cultured in low copper concentration MNK and WND localize to the *trans*Golgi network but in high copper relocalize either to the plasma membrane (MNK) or a vesicular compartment (WND). In this paper we investigate the role of the MBSs in Cu-transport and trafficking. The copper transport activity of MBS mutants of MNK was determined by their ability to complement a strain of *Saccharomyces cerevisiae* deficient in CCC2 (Δccc2), the yeast MNK/WND homologue. Mutants (CXXC to SXXS) of MBS1, MBS6, and MBSs1-3 were able to complement Δccc2 while mutants of MBS4-6, MBS5-6 and all six MBS inactivated the protein. Each of the inactive mutants also failed to display Cu-induced trafficking suggesting a correlation between trafficking and transport activity. A similar correlation was found with mutants of MNK in which various MBSs were deleted, but two constructs with deletion of MBS5-6 were unable to traffic despite retaining 25% of copper transport activity. Chimeras in which the N-terminal MBSs of MNK were replaced with the corresponding MBSs of WND were used to investigate the region of the molecules that is responsible for the difference in Cu-trafficking of MNK and WND. The chimera which included the complete WND N-terminus localized to a vesicular compartment, similar to WND in elevated copper. Deletions of various MBSs of the WND N-terminus in the chimera indicate that a targeting signal in the region of MBS6 directs either WND/MNK or WND to a vesicular compartment of the cell.

Introduction

Copper homeostasis is the process by which organisms maintain adequate supplies of copper to essential cuproenzymes, and excrete or sequester excess amounts of this potentially toxic element. At a systemic level in mammals, copper supplies are maintained by a balance between rate of uptake of dietary copper in the small intestine and the rate of excretion of copper in the bile. The amount of copper absorbed from food has been found to depend on the copper content of the diet: when the dietary copper intake is very low, a high percentage is absorbed while the amount absorbed is reduced as the copper content of the diet increases (Turnlund *et al.* 1998). The liver is the central organ in copper homeostasis and rapidly removes most of the absorbed copper from the blood. The regulation of copper status in the hepatocyte is achieved by secretion of copper as ceruloplasmin or by excretion of excess copper in the bile. If the amount of copper in the hepatocyte is excessive, copper is excreted in the bile which is the main regulatory step in maintaining overall copper homeostasis (Linder 1991). The systemic processes involved in copper transport has been well summarized in a number of reviews (Linder 1991; Danks 1995; Linder *et al.* 1999). More recently, reviews incorporating the molecular advances have appeared (Vulpe & Packman 1995; Pena *et al.* 1999; Harris 2000).

The effects of disruption of these homeostatic mechanisms are illustrated by the genetic disorders of copper homeostasis, Menkes and Wilson diseases (Danks 1995). Menkes disease is a genetic copper deficiency, which is due to defective uptake and distribution of copper, and Wilson disease is a copper toxicosis condition, due to defective biliary excretion of copper. Identification of the gene affected in Menkes disease was reported in 1993 (Chelly *et al*. 1993; Mercer *et al*. 1993; Vulpe *et al*. 1993), and subsequently led to the isolation of the gene affected in Wilson disease, as the two genes encode closely related copper ATPases with approximately 67% amino acid identity (Bull *et al*. 1993; Tanzi *et al*. 1993; Yamaguchi *et al*. 1993). The Menkes protein is referred to as MNK or ATP7A and the Wilson protein is WND or ATP7B. Both proteins are members of the P-type ATPase family of cation transporters, which include transmembrane domains that form a channel through a cell membrane, an ATP binding site and an invariant aspartic acid that is reversibly phosphorylated during the reaction cycle (Kuhlbrandt *et al*. 1998). MNK and WND are the first heavy metal transporting ATPases identified in mammals. Heavy metal P-type ATPases have additional features that adapt to the transport of heavy metals: they have metal binding sites (MBSs) with a canonical sequence Cys XX Cys, where X is a variable amino acid in the N-terminal region of the protein, and a CysProCys motif in the channel. The cysteines are thought to bind the metal as it traverses the membrane. MNK and WND have the unusual feature of six CXXC motifs in the N-terminal region, which contrasts with the one or two MBSs found in the heavy metal ATPases of the bacteria and the Cu-ATPase in yeast. Figure 1 shows a diagrammatic representation of the structural features of these proteins. The function of the six metal binding sites has been of some interest, and in particular, whether they are involved in regulation of the copper transport activity of these proteins.

Despite the marked difference in clinical phenotypes of Menkes and Wilson diseases, both MNK and WND have similar copper efflux roles in the cell. The different clinical phenotypes resulting from mutation of these genes can be explained by their distinct pattern of expression, as MNK is expressed in most tissues except in the liver, and WND is expressed mainly in the liver. Both proteins deliver copper into the secretory pathway for incorporation of copper into secreted cuproenzymes such as lysyl oxidase (MNK) and ceruloplasmin (WND). In addition, they are the key cellular copper efflux molecules. Efflux of copper from cells forming various epithelial barriers such as the intestinal enterocytes, and the blood brain barrier is an important role for MNK, and efflux of copper from hepatocytes into the biliary canaliculae is a pivotal role for WND. Thus these two molecules play a central role in physiological copper homeostasis

Both MNK and WND exhibit the phenomenon of Cu-induced trafficking, a process by which the intracellular location of the proteins is influenced by the concentration of copper in the cytoplasm. In cultured cells exposed to normal copper concentrations both proteins are located in the *trans*Golgi network, consistent with their role in supplying copper to secreted cuproenzymes. When the cells are exposed to high copper, MNK and WND relocalize to either the plasma membrane (MNK) (Petris *et al*. 1996) or a vesicular compartment (WND) (Hung *et al*. 1997). In polarized hepatocytes, WND traffics first to the vesicular compartment and then to the apical surface of the cell (Roelofsen *et al*. 2000). It is likely that copper-induced trafficking underlies the regulation of copper absorption and excretion, and is a key mechanism in the physiological regulation of copper. Thus there has been considerable interest in the mechanism by which copper causes the relocation of these proteins. Our previous work demonstrated that only one MBS, number five or six in MNK was necessary for Cu-induced trafficking (Strausak *et al*. 1999), if both MBS5-6 are mutated, no Cu-induced trafficking occurs. Goodyer *et al.* reported, however, that any one of the metal binding sites was sufficient for trafficking (Goodyer *et al*. 1999). The copper transport activity of the mutant proteins was not determined. Studies with WND have demonstrated that MBS6 alone is sufficient for copper transport activity, but the trafficking activity of the proteins was not determined (Forbes *et al*. 1999). Other studies have shown that trafficking can be prevented by mutations in other regions of the proteins. A missense mutation causing mild Menkes disease results in a protein that remains in the *trans*Golgi network even in elevated copper (Ambrosini & Mercer 1999) and the mutant MNK found in the mouse homologue of Menkes disease, the brindled mouse, also does not traffic (La Fontaine *et al*. 1999).

Similar results for WND were reported by Forbes *et al.* who found that a number of mutations blocked trafficking of WND (Forbes & Cox 2000) and in addition these mutations also reduced or abolished the copper transport activity of the protein (Forbes & Cox 1998). They considered that these mutations

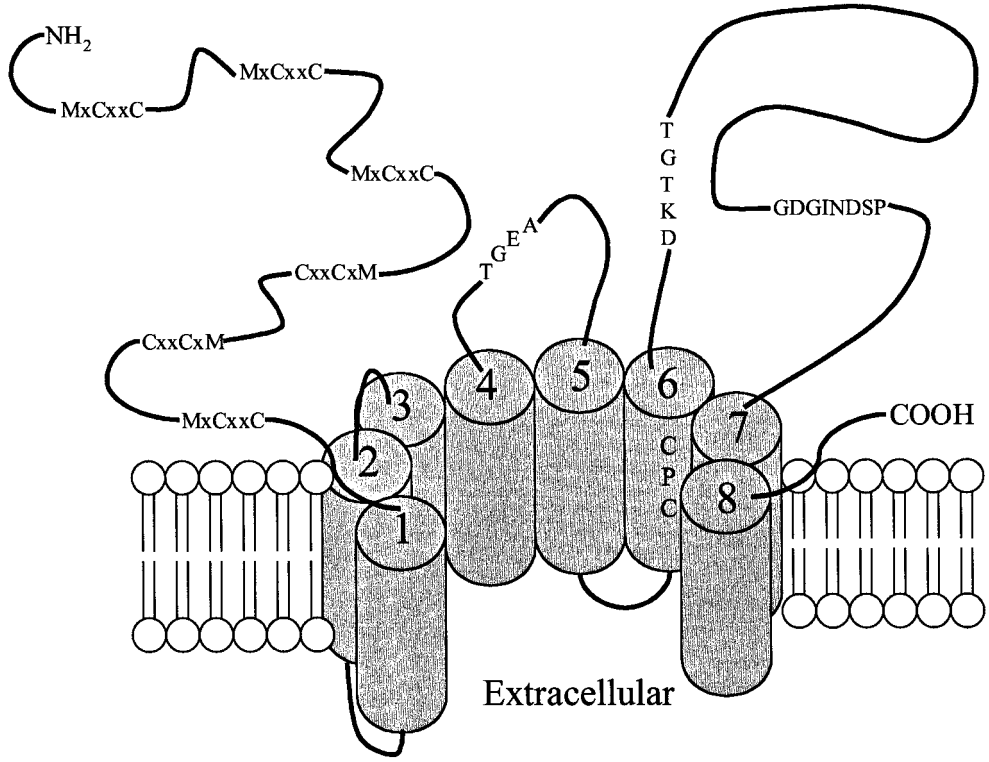

Fig. 1. Proposed topological model of a copper P-type ATPase. N-terminal metal binding sites are indicated by conserved MxCxxC sequences. Other conserved motifs include phosphatase domain (TGEA), transduction domain (CPC), phosphorylation site (DKTGT) and ATP-binding site (GDGINDSP).

might prevent the molecule adopting a conformation required for trafficking to occur (Forbes & Cox 2000).

In this paper we present further data that suggests there is a link between the Cu transport activity of the Cu-ATPases and their ability of traffic, consistent with the idea that a particular conformation is needed for trafficking. In addition we use chimeras between WND and MNK to demonstrate that the region responsible for the different trafficking behaviour of WND and MNK resides in the N-terminal metal binding site regions, located near MBS 6.

Material and methods

Antibody Production

The affinity-purified antibody produced against the first 590 amino acids of the MNK N-terminus was described previously (La Fontaine *et al*. 1998b). Rabbit antibodies directed against the C-terminal peptide with the amino acid sequence RNSPRLGLLDRIVNYSRASIC were produced to detect MNK deleted at the N-terminus. The antibody produced against 275 N-terminal amino acids of Wnd was described previously (La Fontaine *et al*. 2001).

Plasmid constructions

Construction of N-terminal metal binding sites (MBS) mutants MNKm1, MNKm1-3, MNKm6, MNKm5-6, MNKm4-6, MNKm1-6, MNKΔ1-4, MNKΔ1-4m6, MNKΔ1-4m5 were previously described (Strausak *et al*. 1999). MNKΔ5-6 and MNKΔ3-6 were generated using PCR deletion mutagenesis. An in-frame deletion of MBS5-6 was produced by cloning a PCR product of 410 base pairs amplified using pCMB19 as the template. The forward primer was located upstream of the unique *Sph*I site in the MNK cDNA sequence and the reverse primer (40 mer) containing a *Ksp*I site designed to delete amino acids 422-602. The resulting PCR product was cloned into the *Sph*I/*Ksp*I restriction sites of pCMB19 (La Fontaine *et al*. 1998a). The in-frame deletion of MBS3-6 was engineered by cloning a PCR product of 650 base pairs into pCMB19. The PCR product was am-

plified using a forward primer (50-mer) containing the *Sph*I site designed to delete amino acids 285-656 and a reverse primer down stream of the unique *Eco*RV site, and pCMB19 as the template. The PCR product was cloned into pCMB19 using *Sph*I/*Eco*RV sites. After sequence analysis (BigDye^{TM} Terminator Cycle Sequencing Ready Reaction ABI PRISM, PE Applied BIosystems), the mutant MNK cDNA was subcloned into the mammalian expression vector pCMB77 (Petris *et al*. 1998) using *Xba*I/*Sal*I restriction sites. To generate the chimeric protein containing the six metal binding sites of WND and the C-terminal region of MNK (WND1-6MNK), a *Hin*dIII restriction site was introduced by site-directed mutagenesis into the mouse WND cDNA sequence between MBS6 and TM1, resulting in plasmid pCMB162. Mutagenesis was essentially carried out according to the manufacturer's description using the Altered Sites II *in vitro* mutagenesis kit (Promega). Restriction analysis was initially used to verify the introduction of the mutation and subsequently confirmed by manual and automated DNA sequencing using the ThermoSequenase cycle sequencing kit and the dideoxy chain termination kit, respectively (both from Amersham). The 2.8 kb *Hin*dIII fragment of pCMB19 was cloned into pCMB162 to replace the C-terminal region of WND with that of MNK, generating plasmid pCMB171. WNDΔ1-2MNK and WNDΔ1-5MNK were generated by restriction digest analyses of pCMB196 to remove amino acid residues 3-206 and 3-535, respectively, and their construction will be detailed in full in a future publication.

Cell culture and transient transfections

Cell culture conditions were as described previously (Camakaris *et al*. 1995). CHO-K1 cells were seeded onto 13 mm coverslips on the day before the experiment to approximately 60% confluence. For each transfection, 0.5–1 μg of supercoiled plasmid DNA and 3 μl of LipofectAMINE (Life Technologies, Inc.) was mixed in serum-free BME according to the manufacturer's instructions. Cells were allowed to recover overnight in BME containing 10% fetal calf serum for approximately 18 h after transfection to allow for the expression of the cloned protein. $CuCl_2$ was added to a final concentration of 200 μM, and cells were further incubated for 3 h at 37 °C to study the effect of copper on the localization of the MNK protein. Cells were then fixed in 4% formaldehyde and processed for immunofluorescence analysis as described previously (Petris *et al*. 1996).

Immunofluorescence microscopy

Fixed cells were blocked and immunolabelled as described previously (Petris *et al*. 1996). Primary antibodies consisted of either anti-MNK N-terminus antibodies or anti-MNK C terminus antibodies. The secondary antibody was Alexa Fluor 488 goat anti-rabbit IgG-conjugate (Molecular Probes). Cells were analyzed using a X 60 objective on a Olympus AX70 microscope.

Yeast strains and procedures

The yeast strain *S. cerevisiae* deficient in *CCC2* (Δ*ccc2*) (Fu *et al*. 1995) and derivatives were grown at 30 °C in yeast nitrogen base (YNB, Bio101) supplemented with 2% glucose and peptone (YPD) or the appropriate drop out medium (DOB-URA). For expression of MNK and mutants in yeast, the inserts in the mammalian expression vector pCMB77 were excised with *Bam*HI and cloned into the *Bam*HI site of pVT103U (Vernet *et al*. 1987). Yeast protein extracts were prepared using the TCA method described in Clontech's Yeast Protocols Handbook (PT3024-1). Western blot analysis of was carried out essentially as described previously (La Fontaine *et al*. 1998a).

Complementation assay

The ability of MNK mutants to complement the yeast *S. cerevisiae* defective in CCC2 (Δ*ccc2),* the yeast homologue of MNK and WND, was evaluated on yeast nitrogen base medium lacking uracil and iron. Δccc2 yeast are defective in high affinity iron transport due to the lack of copper incorporation into the multicopper oxidase Fet3, the homologue of ceruloplasmin (Askwith *et al*. 1994; Yuan *et al*. 1995). The assay was adapted from (Payne & Gitlin 1998) and (Forbes & Cox 1998). Briefly, a colony was inoculated into 10 ml DOB-URA and grown overnight at 30 °C. The cells were washed twice in pure water and resuspended in iron-limiting medium (DOB-URA, supplemented with 50 mM MES, pH 6.1 and 250 uM ferrozine) and grown to saturation overnight at 30 °C. The washes were repeated and the cells resuspended in iron-limiting medium, and then diluted to an A_{600} of 0.01. Growth was evaluated at 0, 15, 17, 19, 21, 23 and 39 hours and the growth rate was determined over the linear exponential period.

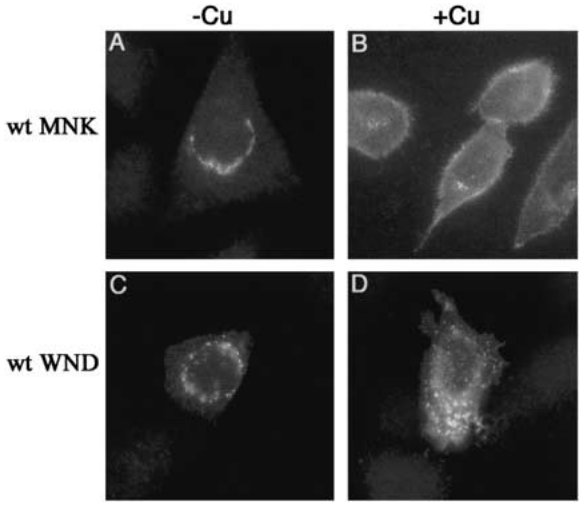

Fig. 2. Effect of copper on wt MNK (A&B) and wt WND (C&D) localization in transiently transfected CHO-K1 cells. Cells transiently transfected with cDNA were cultured for 3 h in normal media (-Cu) or in media containing 200 μM $CuCl_2$ (+Cu). Cells were then fixed, blocked and immunolabelled using a sulfate precipitated fraction C-terminal anti-MNK antibody for wt MNK, and an N-terminal sulfate precipitated fraction anti-WND for wt WND.

Results

To investigate the trafficking behaviour of MNK and WND we prepared cDNA constructs in which both proteins were expressed from the CMV promoter as previously described (La Fontaine *et al*. 1998b). Figure 2 shows the differences in trafficking displayed by the two Cu-ATPases when expressed in CHO cells. In low copper (Figure 2A & C) both proteins were located in a perinuclear region, consistent with *trans*Golgi localization, as previously reported (Petris *et al*. 1996; Hung *et al*. 1997). Upon exposure to 200 μM copper as copper chloride for 3 h, MNK was found on the plasma membrane (Figure 2B) and WND was in large vesicular structures (Figure 2D). We did not observe significant amounts of WND on the plasma membrane.

To extend our previous studies which demonstrated that only one of metal binding site 5 or 6 was required for the trafficking response to copper (Strausak *et al*. 1999), additional mutations of the N-terminal metal binding sites regions of MNK were prepared. Trafficking studies were carried out as shown in Figure 2 to determine whether the mutants would relocalize in response to copper. In addition, the yeast complementation assay as described in *Materials and methods* was used to determine the copper transport activity of the proteins. Figure 3 shows the range of mutants that we studied. The trafficking behaviour of the initial series of mutants in which the metal binding sites were modified by mutation of the two cysteines to serines (CXXC to SXXS) and the MNKΔ1-4 deletions has been previously reported (Strausak *et al*. 1999), but the Cu transport activity of these molecules has not been previously studied.

Table 1. Trafficking ability and copper transport activity of MNK full length mutant proteins containing CxxC to SxxS mutations.

Construct	Trafficking[a]	Activity%[b]
MNKm1	Yes	94
MNKm6	Yes	102
MNKm1-3	Yes	103
MNKm1-6	No	<2
MNKm4-6	No	<2
MNKm5-6	No	<2

[a]For localization studies, cells transiently transfected with DNA encoding wt and mutant MNKs were cultured for 3 h in normal media (−Cu) or in media containing 200 μM $CuCl_2$ (+Cu). Cells were then fixed, blocked, and immunolabelled using an N-terminal anti-MNK antibody.
[b]Growth rates of wt and mutant MNKs were evaluated as described in Material and Methods and expressed as a percentage of wt growth. Average rates were calculated from four independent experiments.

The trafficking ability and the copper transport activity of the SXXS series of mutants is summarized in Table 1. Mutation of either MBS1 or MBS6 alone did not affect trafficking or the ability of the molecules to complement the growth of the ΔCCC2 yeast strain in iron limiting media; growth rates were close to 100% of those achieved with the wild type construct. Similarly mutation of the first three metal binding sites (MNKm1-3) did not reduce trafficking or transport of copper. However, if all MBS were mutated (MNKm1-6) the molecule did not traffic and could not transport copper. The inactivation of both processes was also observed when MBS4-6 and MBS5-6 were mutated. These results suggest that the ability of MNK to traffic in response to, and transport, copper were linked. In addition the presence of functional MBSs close to the transmembrane domains appear to be required for both processes to occur.

To extend these observations deletion mutants were prepared as shown in Figure 3. The trafficking response and copper transport appeared to be the same as wild type when the first four metal binding domains were deleted (Table 2). Even with only one metal binding site remaining, either MBS5 or MBS6,

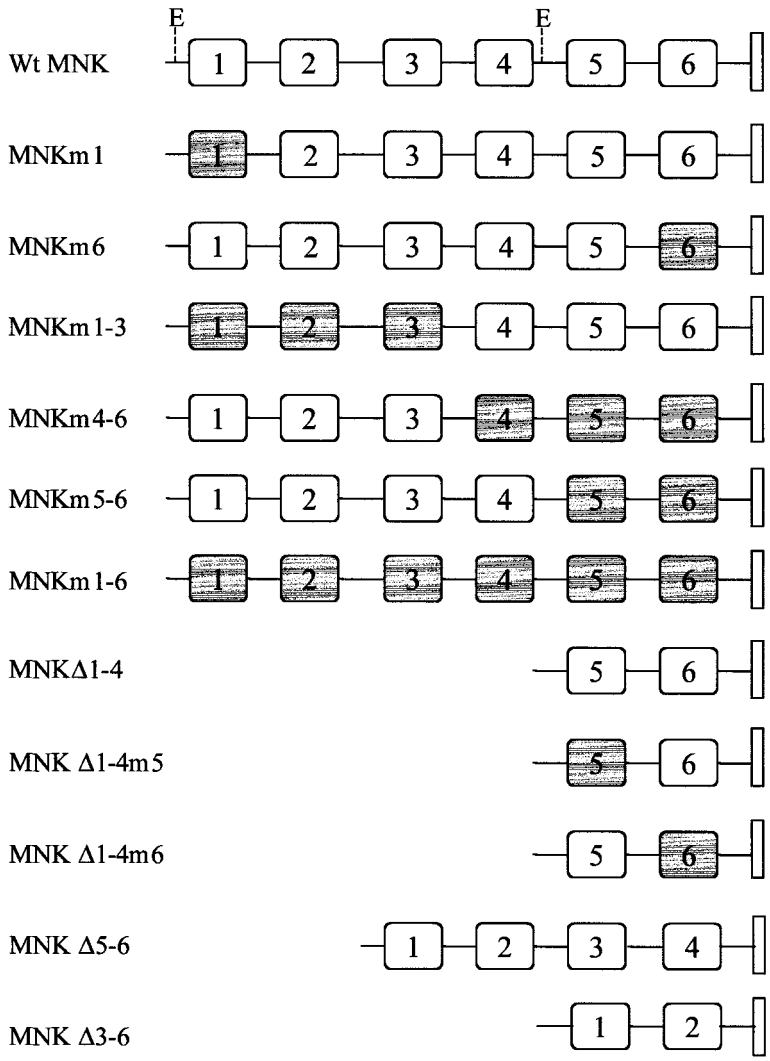

Fig. 3. Schematic representation of mutations and deletions introduced into MNK. As described previously (Strausak *et al*. 1999), MBSs of MNK were mutated from CxxC to SxxS by site directed mutagenesis. White boxes denote wt MBSs and grey boxes indicate mutated MBSs. E, *Eco*RI restriction sites used to delete the first 478 amino acids of MNK. Deletion constructs MNKΔ3-6 and MNKΔ5-6 were generated using PCR deletion mutagenesis. MNKΔ3-6 lacks amino acids 285-656 and MNKΔ5-6 lacks amino acids 422-602.

trafficking occurred as in the wild type (Strausak *et al*., 1999) and copper transport activity was still considerable, although somewhat reduced compared with wild type. Interestingly, the MNKΔ3-6, in which metal binding sites 1&2 replace 5&6, and MNKΔ5-6, in which metal binding site 3&4 replace 5&6, did not exhibit any trafficking, but retained about 25% of copper transport activity. These results suggest that the MBS5/6 region has some important function in trafficking that cannot be substituted for by the other metal binding sites, but that other MBS can substitute, although less effectively, for MBS5-6 for Cu transport.

The above results suggest that the presence of MBS5-6 in the Menkes protein is necessary for Cu-induced trafficking to the plasma membrane to occur. The Wilson protein, however, traffics to a vesicular compartment (Figure 2D). The differential targeting of these molecules could be due to a targeting motif, which determines their destination in high copper. To investigate the region of the molecules that is responsible for the difference in trafficking, we formed chimeras in which the N-terminal 6 MBS of MNK was replaced with the corresponding MBS of WND (Figure 4). The chimeric molecule (WND1-6MNK) was found to be localized in the TGN in low cop-

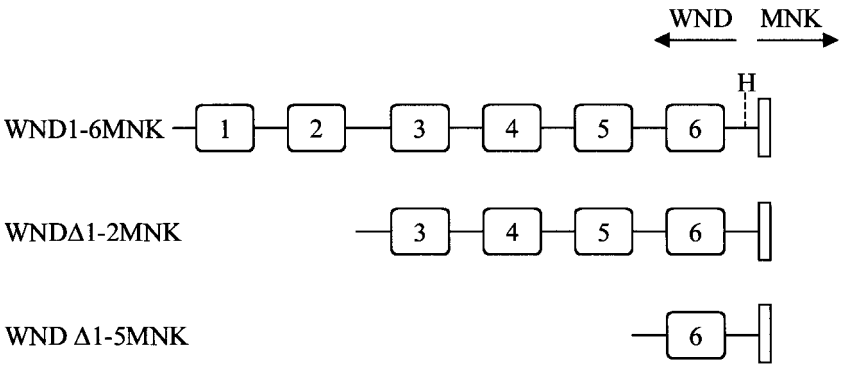

Fig. 4. Schematic representation of WND/MNK chimera and deletion proteins. N-terminus of WND was fused with MNK using a *Hin*dIII site (H) introduced into the mouse WND cDNA between MBS6 and TM1 by site-directed mutagenesis as described under *Material and methods.* Upstream of *Hin*dIII is WND, downstream of *Hin*dIII is MNK. Deletion constructs WNDΔ1-2MNK and WNDΔ1-5MNK were generated by restriction digest of the WND/MNK chimera. WNDΔ1-2MNK lacks amino acids 3-206 and WNDΔ1-5MNK lacks amino acids 3-535.

Table 2. Trafficking ability and copper transport activity of MNK deletion constructs.

Construct	Trafficking[a]	Activity%[b]
MNKΔ1-4	Yes	92
MNKΔ1-4m5	Yes	43*
MNKΔ1-4m6	Yes	85
MNKΔ3-6	No	25
MNKΔ5-6	No	25

[a]For localization studies, cells transiently transfected with DNA encoding wt and mutant MNKs were cultured for 3 h in normal media (−Cu) or in media containing 200 μM $CuCl_2$ (+Cu). Cells were then fixed, blocked, and immunolabelled using a C-terminal anti-MNK antibody.
[b]Growth rates of wt and mutant MNKs were evaluated as described in Material and Methods and expressed as a percentage of wt growth. Average rates were calculated from four independent experiments.
*The reduced transport activity of MNKΔ1-4m5 construct in △CCC2 may be due to the reduced expression of the mutant protein as determined by Western blot analysis (data not shown).

Table 3. Summary of localization data for WND/MNK chimera and deletion chimera proteins.

	Localization	
Construct	(−Cu)	(+Cu)
Wt WND	TGN	Vesicles
WNDm1-6MNK	TGN	Vesicles
WNDΔ1-2MNK	TGN	Vesicles
WNDΔ1-5MNK	TGN	Vesicles
Δ1-6MNK	TGN	TGN

Cells transiently transfected with DNA encoding WND/MNK and deletion chimeras were cultured for 3 h in normal media (−Cu) or in media containing 200 μM $CuCl_2$ (+Cu). Cells were then fixed, blocked, and immunolabelled using a sulfate precipitated fraction C-terminal anti-MNK antibody. TGN, *trans*Golgi network.

per, but on vesicles in high copper (Figure 5), the pattern was very similar to WND in high copper (Figure 2D). This suggests that the targeting information for WND is contained in the N-terminal region. The candidate signal was further localized by deleting portions of the WND N-terminal region, as shown diagrammatically in Figure 4. As can be seen from Table 3, mutants with deletion of the first two MBS of WND (WNDΔ1-2MNK), or even the first five MBS (WNDΔ1-5MNK), still trafficked to the vesicles in a manner similar to wild type WND. However, if all the MBS were deleted, the molecule was unable to traffic (Δ1-6MNK) (Table 3). These results are consistent with the presence of a targeting signal in the region of MBS6 that can direct either MNK or WND to a vesicular compartment of the cell.

Discussion

We consider that Cu-induced trafficking of the Cu-ATPases is a central mechanism that is responsible for overall maintenance of copper status in the body. The elucidation of the molecular basis of this phenomenon is therefore of considerable interest and importance. In previous work we have demonstrated that the N-terminal MBSs play a role in Cu induced trafficking and only one of the two metal binding sites closest to the channel were required for trafficking to occur (Strausak *et al*. 1999). In this paper we have extended

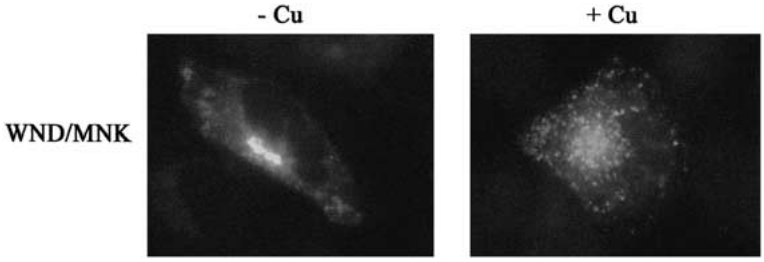

Fig. 5. Effect of copper on WND/MNK localization in transiently transfected CHO-K1 cells expressing WND/MNK chimera. Cells transiently transfected with DNA encoding WND/MNK chimera were cultured for 3 h in normal media (−Cu) or in media containing 200 μM $CuCl_2$ (+Cu). Cells were then fixed, blocked, and immunolabelled using a sulfate precipitated fraction C-terminal anti-MNK antibody.

these observations and relate the trafficking behaviour to Cu-transport ability of the mutant molecules. In the first series of MNK mutants the cysteines in the MBSs were altered to serines, thus abolishing copper binding, demonstrating that molecules that had no copper transport activity also did not traffic (Table 1). Of interest is the fact that MNKm1-3 had normal Cu transport activity. This contrasts with the results of Payne and Gitlin (Payne & Gitlin 1998) who found progressive mutation of the MBSs in MNK from CXXC to SXXS resulted in loss of copper transport activity after the second MBS had been mutated. Thus their corresponding construct of MNKm1-3 was inactive, yet we find this molecule to have 100% activity, in agreement with the equivalent analysis of the effect of mutation of the MBSs in WND by Forbes and Cox (Forbes *et al*. 1999). This discrepancy between results with WND and MNK led Forbes and Cox to propose that there may be a fundamental difference between the mechanism of action of the two Cu-ATPases. Our results suggest that this is not the case.

The results of the deletion constructs (Table 2) demonstrate that not all molecules that are capable of transporting copper have the ability to traffic, and that the region of MNK that includes MBS5-6 appears to have a critical role in trafficking. The deletions MNKΔ3-6 and MNKΔ5-6 are of particular interest, in that they retain a significant transport activity (about 25%) and yet are completely unable to traffic. This result demonstrates that replacement of MBS5-6 by the structurally similar MBS1-2 (in MNKΔ3-6) or MBS3-4 (in MNKΔ5-6) allows some Cu transport, but not trafficking. The exact roles of the multiple MBSs are not clear, but it is reasonable to propose that copper is delivered to the MBSs closest to the N-terminus by the copper chaperone ATOX1. Indeed there is some specificity of interaction of ATOX1 to the MBSs, a higher binding was reported with the outer MBSs, and no interaction with MBS5-6 of WND could be detected (Larin *et al*. 1999). The inner MBSs (MBS5-6) may receive copper from the outer MBSs and then transfer the metal to the CPC motif in the channel. If this is the role of the inner MBSs, then our data suggests that the outer MBSs are possibly capable of performing this function, although with a lower efficiency.

From these results it is unclear why MBS5-6 are critical for trafficking, but some clue to this function came from the chimera experiments. As shown in Figure 2 and previously reported, MNK and WND are targeted differently in cells in response to copper (Petris *et al*. 1996; Hung *et al*. 1997). MNK moves to the plasma membrane and WND to large vesicles that resemble late endosomes (La Fontaine *et al*. 2001). In polarized cells, such as the hepatocyte, WND moves beyond the vesicle to the apical surface of the cell (Roelofsen *et al*. 2000). It is likely that MNK targets the basolateral surface of polarized epithelial cells, because in the intestinal enterocytes of patients with Menkes disease copper accumulates in the enterocyte and the transfer of copper into the circulation across the basolateral surface is blocked (Danks & Cartwright 1973). We investigated the molecular basis of the differential targeting of MNK and WND by replacing the N-terminus of MNK with that of WND (WND1-6MNK). This exchange converted MNK into a vesicular targeted molecule, trafficking in a manner indistinguishable from WND. Furthermore, the critical region was narrowed down to a region around MBS6, as WNDΔ1-5MNK was also capable of trafficking to vesicles, but Δ1-6MNK could not traffic. This result can be interpreted as indicating that an apical targeting signal is present in the region of WND MBS6. If so the failure of MNKΔ3-6 and MNKΔ5-6 to traffic even though they can transport

copper is due to the absence of the putative targeting signal.

In conclusion we consider that the Cu-induced trafficking of MNK and WND to be both dependent on the molecule having copper transport activity and the presence of a targeting signal, which for WND at least, is located around MBS6. The dependence of trafficking on Cu-transport is consistent with the suggestion of Forbes and Cox (Forbes & Cox 2000) that a specific conformation of the ATPases is required for the trafficking. This conformation may be one of the recognized E1-E2 conformations that occur during the transport of cations by P-type ATPases (Jencks 1992).

Acknowledgements

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