

# The Different Intermolecular Interactions of the Soluble Copper-binding Domains of the Menkes Protein, ATP7A\*<sup>§</sup>

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ATP7A is a P-type ATPase involved in copper(I) homeostasis in humans. It possesses a long N-terminal cytosolic tail containing six domains that are individually folded and capable of binding one copper(I) ion each. We investigated the entire N-terminal tail (MNK1–6) in solution by NMR spectroscopy and addressed its interaction with copper(I) and with copper(I)-HAH1, the physiological partner of ATP7A. At copper(I)-HAH1:MNK1–6 ratios of up to 3:1, thus encompassing the range of protein ratios *in vivo*, both the first and fourth domain of the tail formed a metal-mediated adduct with HAH1 whereas the sixth domain was simultaneously able to partly remove copper(I) from HAH1. These processes are not dependent on one another. In particular, formation of the adducts is not necessary for copper(I) transfer from HAH1 to the sixth domain. The present data, together with available *in vivo* studies, suggest that the localization of ATP7A between the trans-Golgi network and the plasma membrane may be regulated by the accumulation of the adducts with HAH1, whereas the main role of domains 5 and 6 is to assist copper(I) translocation.

Human ATP7A is a P-type ATPase that receives copper(I) in the cytosol from the metallochaperone HAH1 and translocates it to the trans-Golgi network or across the plasma membrane (1, 2), depending on cellular conditions (3). A second, closely related human copper(I)-transporting P-type ATPase is ATP7B (WLN hereafter). Mutations in ATP7A may lead to a fatal X-linked copper deficiency syndrome (4–6), the Menkes disease. Hence, ATP7A is often referred to as the Menkes protein (MNK hereafter). Mutations in WLN are instead responsible for Wilson disease (7, 8). In Menkes disease, copper transport across membranes is defective, particularly in intestinal mucosal cells where copper efflux is impaired. Indeed, MNK has two important cellular functions: (i) to facilitate the export

of copper from non-hepatic tissues and its absorption into circulation, and (ii) to deliver copper to the secretory pathway for incorporation into copper-dependent enzymes (9). To carry out both functions, MNK must be correctly localized within distinct subcellular compartments, which is achieved through a copper-dependent trafficking of the polypeptide (3). Under low copper conditions, MNK is localized within the trans-Golgi network (TGN)<sup>2</sup>; copper stimulation results in the redistribution of MNK to the plasma membrane (3).

MNK contains four major regions/domains (the N-terminal copper binding tail, the transmembrane domain, the ATP-binding domain, and the phosphatase domain) and a C-terminal tail. The N-terminal copper binding cytosolic tail of MNK is ~650 amino acids long and contains six 70-amino acid independently folded domains, which are relatively similar in sequence and structure (10–16). Each domain harbors the conserved sequence motif GMXCXXC, through which it can bind one equivalent of copper(I) (17). Two outstanding open questions are (i) why mammalian MNK homologs have six metal-binding domains when lower organisms, including yeast, have only one or two, and (ii) whether there is any functional differentiation between these six domains.

Complementation studies in yeast and confocal microscopy studies in mammalian cells have provided extensive information on the activity and trafficking capabilities of MNK variants. The presence of either the intact fifth or intact sixth metal-binding domain is sufficient to support both MNK activity and intracellular trafficking at levels normal or close to normal (18–22). The level of activity when only the fifth domain is intact is ~80% with respect to the wild-type protein; a mutant MNK with only the intact sixth domain has normal activity (18–23). An artificial construct in which domains 3–6 have been removed so that domains 1 and 2 are in the same position with respect to the membrane of domains 5 and 6 in the WT protein has no trafficking ability but supports yeast growth at 25% of normal levels (22). If the copper binding cysteines are mutated to serine, thus abrogating metal binding activity, MNK only localizes to the TGN even in the presence of excess copper (22). In the all Cys-to-Ser mutant, phosphorylation of MNK, which is the key event triggering protein migration from the TGN (24), is still possible albeit at a presumably significantly reduced rate (21). MNK trafficking is also affected by the presence of HAH1, which establishes the copper concentration threshold for MNK relocation (25). In this work, we investigated the interaction

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Backbone resonance assignments of apo- and copper(I)-MNK1–6 are available from the BioMagResBank (codes 15164 for apo-MNK1–6 and 7375 for copper(I)-MNK1–6).

<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1.

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<sup>2</sup> The abbreviations used are: TGN, trans-Golgi network; WT, wild type.

of copper(I) and of copper(I)-HAH1 with the N-terminal tail of MNK (MNK1–6 hereafter) to obtain insights into the different behavior of the six metal-binding domains in the context of the entire cytosolic portion of the protein. These differences form the basis for the comprehension of the atomic level mechanism regulating MNK trafficking.

## EXPERIMENTAL PROCEDURES

**Preparation of Protein Samples**—For MNK1–6 expression, the DNA segment corresponding to residues 5–633 of MNK was amplified by PCR and cloned in the Gateway Entry vector pENTR/TEV/D-TOPO (Invitrogen) to include the TEV protease cleavage site at the N-terminal end. This segment was then subcloned into pDEST 17 (Invitrogen) through the Gateway LR reaction, yielding a plasmid expressing the protein with a His tag at the N terminus. The expression plasmids for all mutants were obtained through the QuikChange site-directed mutagenesis kit (Stratagene).

WT and mutant MNK1–6 were expressed in *Escherichia coli* Rosetta (DE3) cells (Novagen) in minimal medium cultures. For isotope enrichment,  $(^{15}\text{NH}_4)_2\text{SO}_4$  and  $[^{13}\text{C}]\text{glucose}$  were used. The protein product was purified using HisTrap chelating FF columns (GE Healthcare). The His tag was cleaved with AcTEV protease (Invitrogen). Protein purity was checked by SDS-PAGE and matrix-assisted laser desorption ionization time-of-flight mass spectra. Copper(I) was added to samples in an inert atmosphere chamber (Coy Laboratory) by adding an acetonitrile solution of tetrakis(acetonitrile)copper(I) hexafluorophosphate ( $\text{Cu}(\text{CH}_3\text{CN})_4\text{PF}_6$ ) in the presence of 1 mM dithiothreitol. All samples contained between 0.2 and 0.5 mM protein in 50 mM phosphate at pH 7.0.

HAH1 samples were prepared as previously described, always without poly-His tag (26). MNK1 (spanning residues 5–77 of MNK) samples were prepared using the same protocol used for other individual domains (10, 12). In titration experiments we added copper(I)-HAH1 or copper(I)-MNK1 to apo-MNK1–6 directly in the NMR tube under  $\text{N}_2$  atmosphere.

**NMR Spectroscopy**—NMR experiments were acquired using Bruker Avance spectrometers operating at proton frequencies of 500, 800, and 900 MHz, all equipped with cryogenically cooled probes. Resonance assignments of WT MNK1–6 (both apo and copper-loaded) were performed through conventional multidimensional NMR techniques based on triple resonance experiments (27). The backbone dynamics of apo-WT-MNK1–6 was investigated through the analysis of  $^{15}\text{N}$   $R_1$ ,  $R_2$  relaxation rates and heteronuclear  $^1\text{H}$ - $^{15}\text{N}$  nuclear Overhauser effects (28).

Copper(I) binding by WT or mutant MNK1–6 was assessed by titrating the protein with  $\text{Cu}(\text{CH}_3\text{CN})_4\text{PF}_6$  and following the titration through  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectra. Titrations of MNK1–6 with copper(I)-HAH1 or copper(I)-MNK1 were followed in the same way. Protein-protein interaction experiments were performed both with and without dithiothreitol in the solutions, which did not cause significant differences in the results.

**Immunoblotting**—SH-SY5Y neuroblastoma cells and human fibroblast cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and supplemented

with glutamine and penicillin/streptomycin. All cells were maintained in 5%  $\text{CO}_2$  in air in a humidified incubator at 37 °C. Cells were lysed in radioimmune precipitation buffer (NaCl 150 mM, Tris-HCl 50 mM, pH 7, Nonidet P-40 1%, NaF 100 mM, EGTA 2 mM) with 10% glycerol supplemented with protease inhibitors for 20 min at 4 °C followed by centrifugation. Protein concentration for all samples was determined by Bradford's method (Bio-Rad). For HAH1 analysis, lysates (100  $\mu\text{g}/\mu\text{l}$ ) were heated at 100 °C for 10 min in the presence of SDS and 2-mercaptoethanol and then separated by reducing SDS-PAGE gel electrophoresis and transferred to nitrocellulose. The same procedure, but without heating, was used for the analysis of MNK. Membranes were blocked with 5% milk in phosphate-buffered saline for 1 h, incubated overnight with a 1:1000 dilution of primary antibody at 4 °C, and finally washed several times with phosphate-buffered saline and 0.1% Tween 20. Membranes were then incubated with anti-rabbit IgG conjugated to horseradish peroxidase, washed, and developed using enhanced chemiluminescence reagent (Amersham Biosciences) according to the manufacturer's protocol.

## RESULTS

**Interaction of the Cytosolic Tail of MNK with Copper(I) and with Copper(I)-HAH1**—The entire cytosolic tail of MNK, comprising its six copper(I)-binding domains, was expressed in *E. coli* as a single construct. Heterologous expression allowed us to enrich the protein in the  $^{15}\text{N}$  and  $^{13}\text{C}$  stable isotopes. Protein solubility was  $\sim 300 \mu\text{M}$  at pH 7.0. Two-dimensional  $^1\text{H}$ - $^{15}\text{N}$  spectra of MNK1–6 showed a good dispersion of signals (supplemental Fig. S1), which could be assigned through conventional multidimensional NMR techniques (27), thanks to the availability of assignments for the individual domains (10–15, 29). We assigned signals from all six copper(I)-binding domains, whereas only very few signals from the segments of polypeptide chain connecting these domains could be observed. This is presumably because these regions are poorly structured and thus leave backbone amide protons unprotected and ready to exchange with the solvent. A three-domain construct containing domains 4–6 shows similar behavior (29). The analysis of  $^{15}\text{N}$  relaxation rates for the few signals observed from residues in the linker regions showed that the flexibility of these regions uncouples the motion in solution of the various domains with the exception of domains 5 and 6, which are separated by only six amino acids and thus behave more similarly to a single rigid unit. This is also true for domains 5 and 6 of WLN (30).

MNK1–6 could bind copper(I) when presented with  $\text{Cu}(\text{CH}_3\text{CN})_4^+$ , a small inorganic complex that can easily metallate copper(I) binding chaperones or ATPases (31). Copper(I) appeared to distribute over all the six domains (Fig. 1). Using substoichiometric copper(I) concentration, the distribution of the metal ion among the domains was shown to be slow on the NMR time scale (equilibration time lower than ms), as the spectra contained distinct signals for both the apo- and metal-bound forms. The position of the signals from the various domains did not depend on the copper(I):protein ratio, indicating that there were no copper-mediated interactions among the domains. Unfortunately, NMR spectra did not allow us to quan-

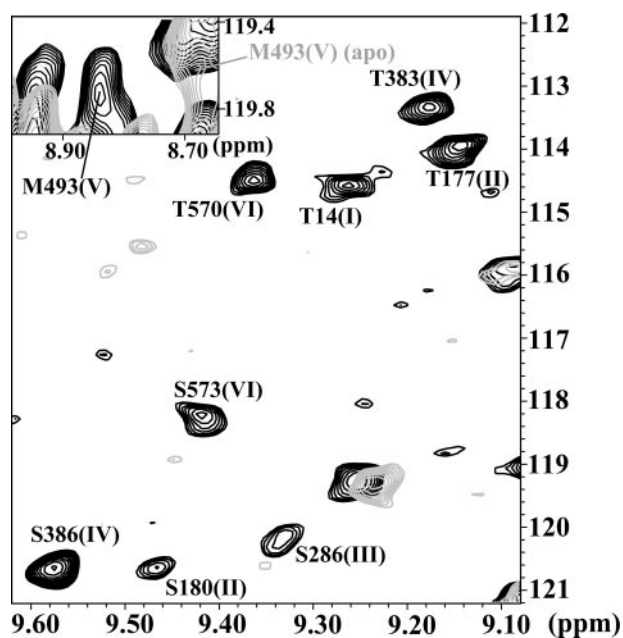


FIGURE 1. **Metallation of MNK1-6 by a copper(I) complex,  $\text{Cu}(\text{CH}_3\text{CN})_4\text{PF}_6$ .** Overlay of selected regions of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of apo-MNK1-6 (gray) and of MNK1-6 in the presence of substoichiometric  $\text{Cu}(\text{CH}_3\text{CN})_4^+$  concentration (black). The protein concentration was always 0.3 mM, whereas in the black spectrum the copper(I) concentration was 0.8 mM. Only signals of the metal binding loop distinctive of the copper(I)-bound form from all the six domains are labeled. In apo-MNK1-6, the signals of the Thr residues shown resonate in a different spectral region ( $^1\text{H}$  shift between 10.1 and 10.8 ppm,  $^{15}\text{N}$  shift between 117 and 123 ppm), whereas the signals of the Ser residues shown are not detectable in the apoprotein even in single-domain constructs. The domain number is given in parentheses (Roman numerals).

titate the relative affinity for copper(I) in the intact MNK tail, because of the weak signal intensities coupled with differential line broadening induced by metallation in the six domains (29). This effect increased with increasing copper(I):protein ratio, leading to the spectrum of fully metallated MNK1-6 being devoid of many signals. Notably, at copper(I):protein ratios of  $\sim 1:1$  (i.e. when only 1/6 of the domains can be metallated), signals from metallated domain 3 were also observed despite its lower affinity for copper(I) when removed from the context of the entire MNK tail (13).

We then checked whether apo-MNK1-6 was able to receive copper(I) from HAH1 and whether there was a differentiation among the six metal-binding domains in their interaction with the metallochaperone. This was done by adding copper(I)-HAH1 to MNK1-6, with one of the two proteins enriched in  $^{15}\text{N}$  so that only its signals appeared in  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra. At a 1:1 copper(I)-HAH1: $^{15}\text{N}$ -MNK1-6 ratio, which corresponds to one equivalent of copper(I) being available for seven binding sites (one on HAH1 and six on MNK1-6), the signals of only domains 1, 4, and 6 experienced a perturbation with respect to the spectrum of MNK1-6 alone (Fig. 2, A and B). In particular, for domain 6 a second set of signals appeared, as observed also for  $\text{Cu}(\text{CH}_3\text{CN})_4^+$ , corresponding to the copper(I)-bound form of this domain (Fig. 2A). Instead, copper(I)-HAH1 caused a variation of the chemical shifts of the signals from the backbone amide moieties of domains 1 and 4 (Fig. 2B), for which a single set of signals was observed throughout the titration.

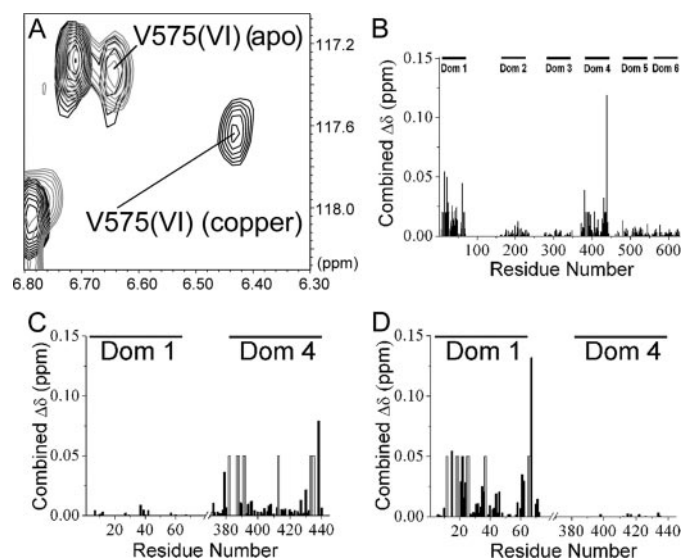


FIGURE 2. **Interaction of WT and mutant apo-MNK1-6 with copper(I)-HAH1 (viewed from the side of MNK1-6).** A, overlay of selected regions of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of apo-MNK1-6 (gray) and apo-MNK1-6 (black) in the presence of three equivalents of copper(I)-HAH1, highlighting the presence of peaks from both the apo- and copper(I)-loaded forms of domain 6. B, combined chemical shift differences between apo-MNK1-6 and apo-MNK1-6 in the presence of three equivalents of copper(I)-HAH1. C, combined chemical shift differences between apo-C1MNK1-6 and apo-C1MNK1-6 in the presence of two equivalents of copper(I)-HAH1. D, combined chemical shift differences between apo-C4MNK1-6 and apo-C4MNK1-6 in the presence of two equivalents of copper(I)-HAH1. In panels B-D, white bars indicate peaks that became broad beyond detection upon interaction, to which, only for graphical representation, a  $\Delta\delta$  value of 0.05 ppm was arbitrarily assigned.

From the above data, it can be inferred that copper(I)-HAH1 formed detectable amounts of a macromolecular complex with both domains 1 and 4, whereas domain 6 removed copper(I) from the metallochaperone, likely through formation of a similar adduct at a concentration too low for detection by NMR. It is possible that HAH1 transfers copper(I) also to domain 5, because the signals of the copper-bound domain 5 are very weak and might have been missed in titration experiments, where broadening occurred for many MNK1-6 signals. Consistent with this picture, the interaction between apo-MNK1-6 and  $^{15}\text{N}$ -copper(I)-HAH1, viewed from the side of the latter, revealed both a variation of the chemical shifts of the signals from the backbone atoms of copper(I)-HAH1 (Fig. 3A) and formation of some apo-HAH1. Mapping of the chemical shift changes experienced by domains 1 and 4 and by HAH1 onto the corresponding solution structures defined the regions of intermolecular contact (Fig. 4).

**Copper(I) and the Metal Binding Cysteines of Domains 1 and 4 Are Necessary for Interaction with HAH1**—To test the hypothesis that domains 1 and 4 of MNK1-6 independently formed an intermolecular adduct with HAH1 through a bridging copper(I) ion, we built several mutants of MNK1-6 in which pairs of cysteines were removed from selected domains and investigated their interaction with copper(I)-HAH1. Hereafter, we will refer to a mutant of MNK1-6 in which the metal binding cysteines of domain  $n$  had been mutated to alanine as  $\text{C}_n\text{MNK1-6}$  (e.g. C1MNK1-6 identifies the mutant lacking the cysteines of domain 1).

In the interaction of C1MNK1-6 with copper(I)-HAH1, we still observed chemical shift variations for residues belonging to

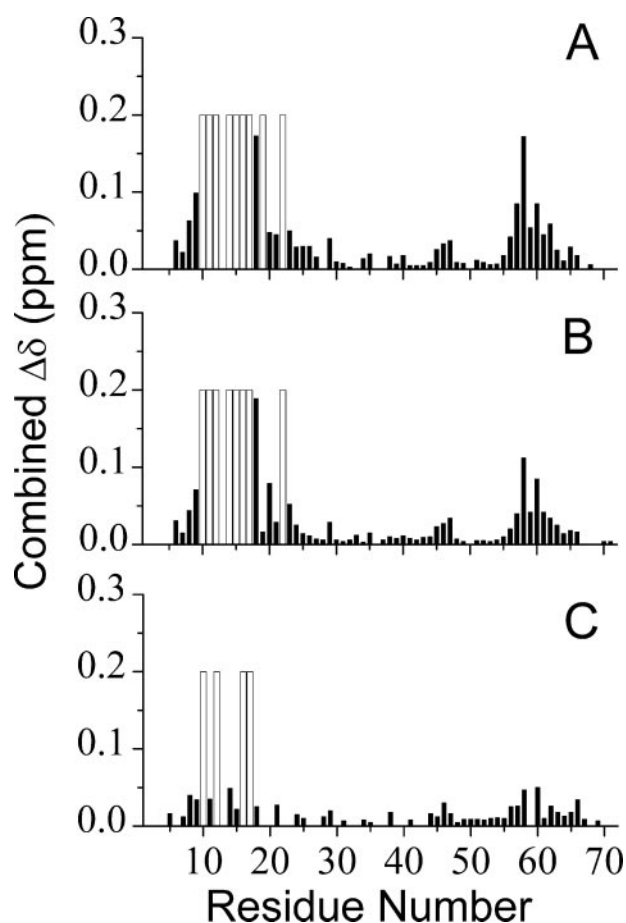


FIGURE 3. Interaction of WT and mutant apo-MNK1-6 with copper(I)-HAH1 (viewed from the side of HAH1). Combined chemical shift differences between copper(I)-HAH1 and copper(I)-HAH1 in the presence of equimolar apo-MNK1-6 (A), apo-C1MNK1-6 (B), and apo-C4MNK1-6 (C). White bars indicate peaks that became broad beyond detection upon interaction, to which, only for graphical representation, a  $\Delta\delta$  value of 0.2 ppm was arbitrarily assigned.

domain 4 (Fig. 2C) as well as the appearance of a new set of signals belonging to metallated domain 6, analogous to what observed for WT-MNK1-6. Instead, signals from residues of domain 1 did not experience any appreciable chemical shift changes, indicating that the two metal binding cysteines are necessary to promote protein-protein association between this domain and copper(I)-HAH1. C4MNK1-6 had a complementary behavior, with domains 1 and 4 swapping their roles with respect to the case of C1MNK1-6 (Fig. 2D). When looking at  $^{15}\text{N}$ -HAH1, chemical shift variations as well as formation of some apoprotein took place upon interaction with either C1MNK1-6 or C4MNK1-6. The chemical shift changes for HAH1 were localized to the same regions in the presence of either MNK1-6 mutant, as well as of WT-MNK1-6, indicating that the area of intermolecular contact on HAH1 was essentially the same in all cases (Fig. 3, B and C).

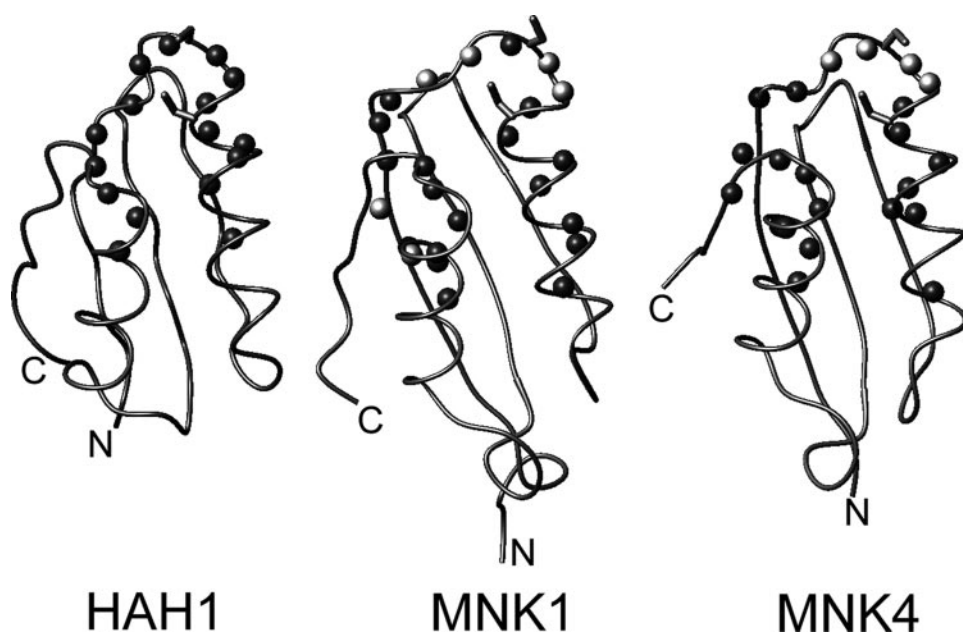
The signals of neither domain 1 nor domain 4 experienced chemical shift variations in WT-MNK1-6 when the latter was titrated with apo-HAH1. Addition of copper(I) at equimolar concentration with respect to HAH1 triggered chemical shift changes. The intermolecular interaction is thus copper-mediated, due to the presence of a bridging copper ion, analogous to the yeast *Atx:Ccc2a* system (32).

**Domain 6 Can Receive Copper(I) Directly from HAH1 or Other Domains**—We then studied the interaction of copper(I)-HAH1 with C1,4MNK1-6 to assess whether copper(I) delivery to the sixth domain from HAH1 must occur sequentially through domains 1 and/or 4 or can be the result of the direct interaction between domain 6 and HAH1 through formation of a reaction intermediate that does not accumulate in the NMR sample, or both. In the former case, C1,4MNK1-6 should be unable to uptake copper(I) in the sixth domain even though the metal-binding site is present. Instead, experiments showed that copper(I)-HAH1 can metallate domain 6 in C1,4MNK1-6 independently of the presence of external thiols, such as dithiothreitol. The signals of HAH1 did not experience chemical shift changes upon interaction with C1,4MNK1-6, whereas the apoprotein formed. Metal transfer thus occurred with a slow kinetics with respect to the NMR time scale, as already observed for WT-MNK1-6.

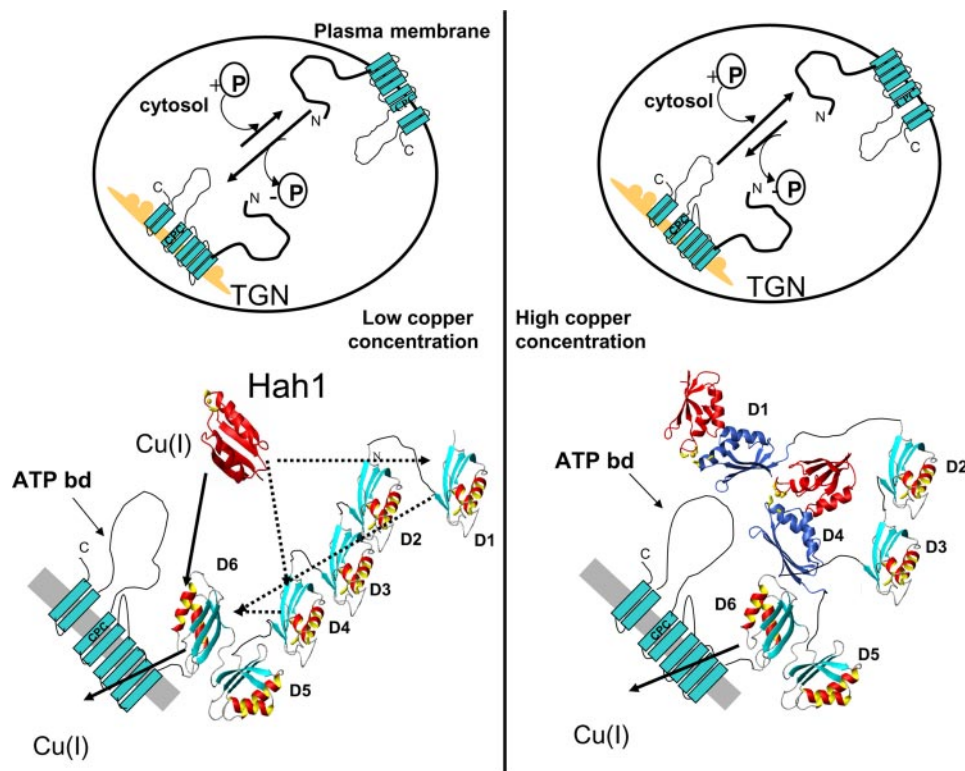
The C1,2,3,4MNK1-6 quadruple mutant could receive copper(I) from copper(I)-HAH1 as well as from copper(I)-MNK1, both of which were able to metallate domains 5 and 6. In a further experiment, we added an equimolar mixture of MNK1 and copper(I)-HAH1 to  $^{15}\text{N}$ -C1,2,3,4MNK1-6, resulting in a 1:1:1:1 copper(I):MNK1:HAH1: $^{15}\text{N}$ -C1,2,3,4MNK1-6 ratio. The aim of this experiment was to verify whether, besides direct transfer of copper(I) to the sixth domain of MNK1-6 from HAH1 or MNK1, a cascade mechanism could also be operative. In this mechanism, free, metallated domain 1 (or domain 4) or HAH1, in equilibrium with the metal-bridged adduct, would transfer the copper(I) ion to domain 6 for subsequent transport across the membrane. Under these conditions, a small fraction of metallated C1,2,3,4MNK1-6 was formed, indicating that the MNK1-copper(I)-HAH1 complex can deliver copper(I) to domain 6 even though it is quite stable with respect to dissociation.

## DISCUSSION

MNK (as well as WLN) interacts specifically with HAH1 to receive copper(I) from it (33, 34). This interaction is needed to sustain copper delivery to cuproenzymes, because the intracellular concentration of free aqueous copper is vanishingly small (35). *In vitro*, the six soluble copper(I)-binding domains of MNK could simultaneously be metallated by  $\text{Cu}(\text{CH}_3\text{CN})_4^+$  (Fig. 1). At substoichiometric  $\text{Cu}(\text{CH}_3\text{CN})_4^+$  concentrations (<six copper(I) ions/protein molecule), all MNK domains were partly metallated, indicating that they have similar affinities for copper(I). An analogous observation has been reported also for the six domains of WLN (34, 36). Similarly to  $\text{Cu}(\text{CH}_3\text{CN})_4^+$ , copper(I)-HAH1 could metallate all six domains of MNK or WLN. The copper(I) affinity of HAH1 is slightly lower than that of the soluble domains of its partners (11, 34), and thus the transfer is thermodynamically favored. However, there was a substantial difference in the behavior of apo-MNK1-6 when presented with substoichiometric quantity of copper(I)-HAH1 instead of  $\text{Cu}(\text{CH}_3\text{CN})_4^+$ . Indeed, copper(I)-HAH1 did not interact equally with all domains, but it formed a copper(I)-bridged intermolecular adduct with both domains 1 and 4 of MNK1-6 and at the same time transferred copper(I) to domain 6 (and probably to domain 5 as well, as suggested by the analysis



**FIGURE 4. Chemical shift perturbation data mapped onto the solution structures of HAH1 (PDB code 1TL5) (26) and of domains 1 (PDB code 1KVI) (15) and 4 (PDB code 1AW0) (14).** The data of Figs. 1 and 2 have been used. Residues featuring a  $\Delta\delta$  value of at least 0.025 ppm (for HAH1) or 0.010 ppm (for domains 1 and 4) are represented as *black spheres*. *Light gray spheres* indicate unassigned residues. The side chains of metal binding cysteines are also shown, and the N and C termini are labeled. For HAH1, residues 9–19, 22, and 57–60 are shown. For MNK1, residues 13–16, 19, 22, 23, 25, 26, 28, 29, 31, 65, 66, 68 are shown in *gray* and 17, 18, 20, 21, and 67 are shown in *white*. For MNK4, residues 385, 386, 391, 392, 394–396, 400, 410, 434, 435, 437–442 are shown in *gray* and residues 387–390 are shown in *white*.



**FIGURE 5. The copper(I)-mediated interaction between HAH1 and MNK protein at different copper concentrations and its relationship with MNK trafficking.** At low metal concentration, HAH1 transfers copper(I) to domains 5 and 6 (*left*). Transitory formation of an adduct between HAH1 and domains 1 and 4 also occurs; the latter two domains may eventually transfer copper(I) to domains 5 and 6 for subsequent translocation across the membrane. At high copper concentration (*right*) the copper(I)-bridged adduct between HAH1 and domains 1 and 4 accumulates and may interact with the ATP-binding domain, affecting the balance between MNK phosphorylation and dephosphorylation in a way such that MNK becomes preferentially localized at the plasma membrane. Copper(I) translocation still occurs through domains 5 and 6.

of the C1,2,3,4MNK1–6 mutant). At 3:1 copper(I)-HAH1:MNK1–6 ratio, domains 2 and 3 essentially did not interact with copper(I)-HAH1 nor were they metallated. Protein quantification using Western blotting indicated the intracellular HAH1:MNK ratios are of the order 1:1–1:2 (not shown), thus well within the range sampled in this work.

Addition of  $\text{Cu}(\text{CH}_3\text{CN})_4^+$  to MNK1–6 results in the appearance of a second set of signals for all domains. The additional signals could be assigned to the copper(I)-bound form of the residues close to the two metal binding cysteines of all six MNK1–6 domains (Fig. 1). In the presence of copper(I)-HAH1 the same feature was observed only for domains 5 and 6 of MNK1–6. Instead, for domains 1 and 4 we observed a variation of the chemical shifts of the signals from residues close to the two binding cysteines and in the loop following the second  $\alpha$ -helix, defining a well distinct protein-protein interaction region (Fig. 4) analogous to what was found for the yeast *Atx1:Ccc2a* system. This suggests that these two domains form an adduct with copper(I)-HAH1 with a configuration similar to that of the homologous yeast system (32, 37).

Domains 1 and 4 of MNK1–6 reacted independently with copper(I)-HAH1. Mutation of the metal binding cysteines to alanine in either of the two domains abrogated its interaction with the partner but left the other domain capable of forming the intermolecular adduct. Both the dependence of the formation on the presence of the metal binding cysteines (Fig. 2, C and D) and the fact that isolated domain 1 interacts with HAH1 only in the presence of copper indicate that the adduct created is a metal-mediated (32) one. By comparing the chemical shift variations measured for HAH1 upon interaction with either C1MNK1–6 or C4MNK1–6, it appears that the adduct formed with domain 4 has a higher formation constant than the adduct formed

with domain 1. Note that metal transfer to domain 6 leaves unaffected the chemical shift values (and other NMR properties) of both apo- and copper(I)-HAH1, as shown previously for isolated domain 6 (10) and here by studying C1,4MNK1–6 and C1,2,3,4MNK1–6, but contributes to determining the distribution of the copper(I) ion among the various binding sites available.

Formation of an intermolecular adduct between copper(I)-HAH1 and domain 4 of WLN had been reported previously (30). In WLN, however, domains 5 and 6 are unable to receive copper(I) from HAH1 and thus the interaction of copper(I)-HAH1 with domain 4 was proposed to be instrumental for metal loading into the latter two domains (30). Here, we observed that both C1,4MNK1–6 and C1,2,3,4MNK1–6 could receive copper(I) directly from the metallochaperone. A mechanism in which copper(I)-HAH1 first forms an adduct with domains 1 or 4, and then the copper(I) ion is transferred by one of these domains to domain 6 or 5, can also contribute.

On the basis of the above data, it is possible to attempt an interpretation of the data available in the literature on cellular studies. The presence in WLN of domain 6 alone is sufficient to complement a  $\Delta ccc2$  yeast (38). The copper transport activity (also measured from complementation experiments in  $\Delta ccc2$  yeast) of MNK having only intact domain 6 is close to normal levels as well (22). The same is true for MNK having only intact domain 5 or having both domains 5 and 6. Normal copper transfer activity of these mutants *in vivo* is fully justified by our present observation that HAH1 can directly transfer the metal ion to domain 6 within the entire cytosolic MNK tail (MNK1–6) and that direct transfer still takes place after the cysteines of domains 1 to 4 are mutated to alanine.

A peculiarity of the MNK and WLN proteins with respect to yeast *ccc2* is that they can traffic from the TGN, respectively, to the plasma membrane or to intracellular vesicles when environmental copper levels are high (3). Thanks to this feature, MNK can carry out two different physiological functions, *i.e.* either exporting copper(I) from intestinal cells or loading copper(I) into the TGN. HAH1 plays an essential role in the process of MNK relocation (25). Intracellular trafficking of MNK and WLN requires formation of an acylphosphate intermediate (24). Formation of this intermediate is partly affected by the cytosolic copper(I)-binding sites and by the intramembrane copper(I)-binding site (CPC), whose presence enhances the efficiency of the trafficking process (21). The localization of MNK or WLN is thus dependent on the balance between the rate of formation of the acylphosphate intermediate, which stimulates trafficking to the plasma membrane or vesicular compartments, and the rate of protein dephosphorylation, which determines back-trafficking from the membrane to the TGN. All these observations can be rationalized by assuming that formation of the copper(I)-bridged adduct of HAH1 with domain 1 or domain 4, or both, is important in determining the balance between forward and back-trafficking (Fig. 5). Indeed, an interaction between the ATP-binding domain of WLN and its cytosolic tail has been demonstrated that can regulate the affinity for ATP of the former (39). The present data indicate that at high copper levels the adduct of HAH1 with one or both domains 1 and 4 of MNK accumulates in the cell. The adduct could contribute to

enhancing the rate of phosphorylation of MNK or stabilize the acylphosphate intermediate with respect to dephosphorylation, or both. By increasing the life time of phosphorylated MNK the copper(I)-dependent macromolecular adduct involving HAH1 and domains 1 or 4 would thus promote the localization of MNK to the plasma membrane, leading to copper(I) export from the cytosolic space. The direct involvement of HAH1 in regulating the trafficking of MNK proposed here is in agreement with the effects of HAH1 knock out observed *in vivo* (25). Additional molecular events may also concur to this same purpose, such as conformational changes or variations of domain-domain dynamics in the cytosolic regions of MNK induced by copper(I) binding to the N-terminal tail (*e.g.* to domains 2 or 3) (13, 18).

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