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Protein kinase-dependent phosphorylation of the Menkes copper P-type ATPase

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Abstract

The Menkes copper-translocating P-type ATPase (ATP7A; MNK) is a key regulator of copper homeostasis in humans. It has a dual role in supplying copper to essential cuproenzymes in the *trans*-Golgi network (TGN) and effluxing copper from the cell. These functions are achieved through copper-regulated trafficking of MNK between the TGN and the plasma membrane. However, the exact mechanism(s) which regulate the localisation and biochemical functions of MNK are still unknown. Here we investigated copper-dependent phosphorylation of MNK by a putative protein kinase(s). We found that in the presence of elevated copper there was a substantial increase in phosphorylation of the wild-type MNK in vivo. The majority of copper-dependent phosphorylation was on serine residues in two phosphopeptides. In contrast, there was no up-regulation of phosphorylation of a non-trafficking MNK mutant with mutated cytosolic copper-binding sites. Our findings suggest a potentially important role of kinase-dependent phosphorylation in the regulation of function of the MNK protein.

Keywords: Copper; Menkes protein; Protein kinase; Phosphorylation

Copper is an essential trace element, whose unique properties make it indispensable for the active sites of enzymes involved in redox reactions such as cytochrome *c* oxidase, lysyl oxidase, and superoxide dismutase. However, excess copper is highly toxic to biological systems through the same redox mechanism that makes it essential to the cell. As a result, a complex mechanism has evolved to allow the delivery of copper to target proteins localised in various compartments and to detoxify the excess amounts of copper by scavenging and efflux from the cell [1].

Two copper transporting P-type ATPases have been shown to play a key role in regulating copper homeostasis in mammals: the Menkes protein (ATP7A; MNK) and the 60% homologous Wilson protein (ATP7B; WND) [2]. The MNK protein, which is expressed in most tissues, except the liver where WND is expressed, has several roles in copper homeostasis: it is essential for systemic copper absorption, it delivers copper to cuproenzymes in the *trans*-Golgi network (TGN) compartment, and facilitates copper efflux when at the plasma membrane (PM) [1]. To perform these tasks in different subcellular compartments, MNK has evolved the ability to traffic from the TGN to the PM and back [3]. There is constitutive recycling between these two compartments [4] and, importantly, copper-regulated relocalisation of MNK to the PM [3].

Several motifs have been identified and characterised as essential for copper-dependent trafficking of MNK. Among these are the N-terminal metal-binding sites (CysXXCys), which play important regulatory roles in copper-induced trafficking and also in catalysis [5,6]. Current information on MNK trafficking suggests that there may be several, potentially interrelated, mechanisms controlling the steady-state localisation and copper-induced trafficking of MNK [2]. Overall it appears

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that copper controls its own homeostasis. While prokaryotic copper P-type ATPases are regulated through copper-dependent transcription factors, this does not appear to play a major role in mammalian cells, where subcellular localisation controlled by copper-responsive trafficking of MNK emerges as a major contributor to copper homeostasis. In eukaryotes, post-translational modifications, particularly protein kinase-dependent phosphorylation, have been shown to be important in regulation of catalytic activity and subcellular localisation of various ATPases, including the Na/K-ATPase, Ca-ATPases, and the cystic fibrosis transmembrane conductance regulator (CFTR) [7–9]. Furthermore, it has been demonstrated that the liver-specific MNK homologue WND undergoes copper-dependent phosphorylation [10].

We hypothesised that the function of MNK may also be regulated by protein kinase-dependent phosphorylation. In the current work we provide evidence that, in the absence of copper, MNK is basally phosphorylated, but the process is rapidly induced upon addition of elevated copper concentrations. This phosphorylation was found to occur exclusively on serine residues and was characterised by increased phosphorylation of specific phosphopeptides and appearance of a novel phosphopeptide. Importantly, phosphorylation of a non-trafficking MNK mutant due to all six metal-binding sites –CxxC– mutated to –SxxS– was not induced in a copper-dependent fashion, although the basal sites were phosphorylated. Our study suggests that copper may exert its self-regulatory homeostatic function at least in part by inducing phosphorylation-dependent changes in the key enzyme, the MNK copper-translocating P-type ATPase.

Materials and methods

Cell culture. Chinese hamster ovary cells (CHO-K1) stably transfected with the wild-type (wtMNK) and the mutant construct (with six putative metal-binding sites –CxxC– mutated to –SxxS–, mMBS) of the Menkes protein cDNA were described earlier [5]. Empty vector transfected CHO-K1 cells (EV) were used as a control in these studies. The growth conditions for the transfected cells were identical. The cells were cultured as a monolayer in Eagle's basal medium with Earle's salts (BME) supplemented with 10% foetal bovine serum (Trace Biosciences, Australia), 2 mM L-glutamine, 0.2 mM proline, 1.2 mM $NaHCO_3$ and 20 mM Hepes in 3% CO_2 at 37 °C.

In vitro phosphorylation. In vitro phosphorylation of MNK using purified membrane vesicles from transfected CHO-K1 cells was performed essentially as described earlier [11] but at 37 °C and in the presence of 60 μM [γ-^{32}P]ATP (200 μCi/ml; Perkin–Elmer).

In vivo phosphorylation. Upon reaching confluency complete BME was replaced with phosphate-free serum-free BME (-P-BME). Following 2 h of incubation the medium was changed again and supplemented with $^{32}P_i$ (Perkin–Elmer; 50 μCi/ml for small-scale experiments; 1 mCi/ml for large-scale experiments) and various concentrations of added copper (0–100 μM) or copper chelators bathocuproinedisulfonate (BCS) and D-penicillamine (100 μM each) for 15–180 min. To study the effect of copper depletion on MNK phosphorylation, the cells were grown in the presence of copper chelators (as above) for 2 days prior to $^{32}P_i$ labelling. To study the turnover or de-phosphorylation of MNK, we conducted pulse-chase experiments, where the cells which were $^{32}P_i$-labelled for 180 min were returned to complete BME (phosphate-sufficient) in the presence or absence of copper for up to 180 min. At the end of the incubation the cells were washed three times with Tris-buffered saline (TBS) buffer (100 mM Tris–HCl, pH 7.5, 150 mM NaCl) and lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 5 mM EGTA, 1% v/v Triton X-100, 10 μg/ml AEBSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 0.2 mM sodium vanadate, 2 mM dithiothreitol (DTT), and 30 mM glycerol phosphate). The lysate was centrifuged at 13,000*g* for 10 min and pre-cleared using Pansorbin (Calbiochem) as a source of protein A for 60 min. Pansorbin was pelleted at 5000*g* for 10 min. The pre-cleared lysate was subsequently incubated with the anti-MNK antiserum [12] for 1 h followed by the addition of fresh Pansorbin beads for 3 h. Pansorbin was pelleted and washed four times with 20 mM MOPS (pH 7.5). At that stage the immunoprecipitated protein could be treated with hydroxylamine or alkaline phosphatase or processed without any treatment. The final pellet was resuspended in the 1× gel loading buffer (Invitrogen) supplemented with 250 mM DTT and 6 M urea and incubated at room temperature (+22 °C) for 30 min. The suspension was centrifuged at 13,000*g* for 10 min and the resultant supernatant was resolved by electrophoresis using 4–12% gradient SDS–PAGE gels (Invitrogen). The gel was dried and radioactive bands were visualised and quantified using a phosphorimager (Typhoon) and ImageQuant software (both Molecular Dynamics, Amersham-Pharmacia), or Kodak Biomax-MS autoradiography film with appropriate intensifying screen (Kodak, Rochester, NY) and a laser densitometer (Molecular Dynamics, Amersham-Pharmacia).

Tryptic phosphopeptide mapping and phosphoamino acid analysis. The radioactive band associated with the Menkes protein was excised from the gel and subjected to in-gel tryptic digestion [13]. Tryptic peptides were sequentially extracted with H_2O and 80% acetonitrile and the combined extracted peptides were lyophilised, resuspended into 5 μl of pH 1.9 electrophoresis buffer containing formic acid:glacial acetic acid:H_2O (50:156:1794 v/v), and spotted onto a cellulose TLC plate. Electrophoresis was for 40 min at 1300 V on a Hunter Thin Layer Peptide Mapping System (CBS Scientific, California) and the TLC plate was then subjected to ascending chromatography in buffer containing glacial acetic acid:pyridine:*n*-butanol:H_2O (15:50:60:75 v/v). Radiolabelled phosphopeptides were visualised after a 24-h exposure using a phosphorimager and ImageQuant software (Molecular Dynamics, Amersham-Pharmacia). Approximately equal cpm of each phosphopeptide was hydrolysed for an hour at 95 °C in 5.7 N HCl. Following addition of unlabelled standards the phosphoamino acids were separated by thin layer electrophoresis at 1000 V for 90 min in pH 2.5 buffer(pyridine:formic acid:glacial acetic acid:H_2O; 1:2.5:17.8:278.7 v/v) and visualised with ninhydrin (0.1% w/v in ethanol) or using a phosphorImager and ImageQuant software (Molecular Dynamics, Amersham-Pharmacia).

Results

In vitro phosphorylation

We demonstrated earlier that, as a part of its catalytic cycle, MNK could undergo copper-dependent transient acyl-phosphorylation at the invariant aspartate residue in the ^{1044}DKTG motif, which is in accordance with the catalytic mechanism for P-type ATPases [11]. Characteristically, the reaction is completed within 20–40 s and the resulting acyl-phosphate is sensitive to hydroxylamine [11]. In this study we used isolated membrane

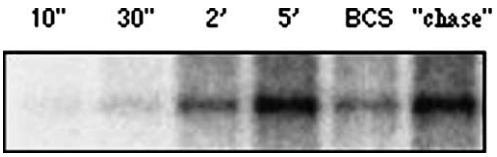

Fig. 1. In vitro phosphorylation of MNK-enriched membrane fraction with [γ-^{32}P]ATP. The reaction was conducted as described in Materials and methods in the presence of 5 μM copper for 10 s, 30 s, 2 min, and 5 min. BCS indicates a 5-min reaction in the presence 1 mM bathocuproinedisulfonic acid. "Chase" indicates the reaction, where upon the completion of 5 min of labelling 1 mM "cold" ATP was added. The reactions were terminated by adding 2% SDS and immunoprecipitated using anti-MNK antisera and proteins were resolved on SDS–PAGE. Shown is the resultant autoradiagram.

vesicles containing the Golgi membranes, the endoplasmic reticulum, and the plasma membrane. We observed rapid phosphorylation upon incubation of isolated MNK-enriched membrane vesicles and [γ-^{32}P]ATP at 37 °C. The initial, low level phosphorylation observed at ≈10–30 s was sensitive to hydroxylamine and could be cleaved with "cold" ATP, consistent with transient acyl-phosphorylation of MNK at the invariant aspartate residue, which has been described in our previous studies [11]. The later, robust phosphorylation was evident by 5 min (Fig. 1) and was largely insensitive to "cold" ATP chase and hydroxylamine (data not shown), suggesting the phosphorylation was not due to acyl-phosphorylation of the invariant aspartate as a part of the catalytic cycle [11]. Importantly, the copper chelator bathocuproinedisulfonate (BCS) significantly reduced the level of phosphorylation without abolishing it completely. These results suggested that MNK was subjected to copper-dependent and copper-independent phosphorylation, presumably catalysed by membrane-associated protein kinase(s). However, to further verify if our observations had in vivo relevance, the phosphorylation studies were extended to whole cells.

In vivo phosphorylation

In vivo metabolic labelling revealed a significant effect of copper on the overall level of MNK phosphorylation. In addition, copper concentration-dependent phosphorylation of wtMNK appeared to be a two-stage process. The first stage was very sensitive to small increases of extracellular copper as there was a significant increase in phosphorylation when the cells were cultured in basal media (0.8 μM copper) compared to copper-depleted conditions following BCS treatment (Fig. 2A, inset). The next significant increase in the level of MNK phosphorylation was observed following the addition of >20 μM copper (Fig. 2A). At 100 μM copper, MNK phosphorylation was elevated 4.6 ± 0.2-fold (mean ± SD) over the levels of phosphorylation in copper chelator-treated cells. In contrast to wild-type MNK, the mutant MNK with all six metal-binding sites (MBSs) mutated (mMBS) showed a relatively small increase in phosphorylation in response to increased copper concentrations (Fig. 2A).

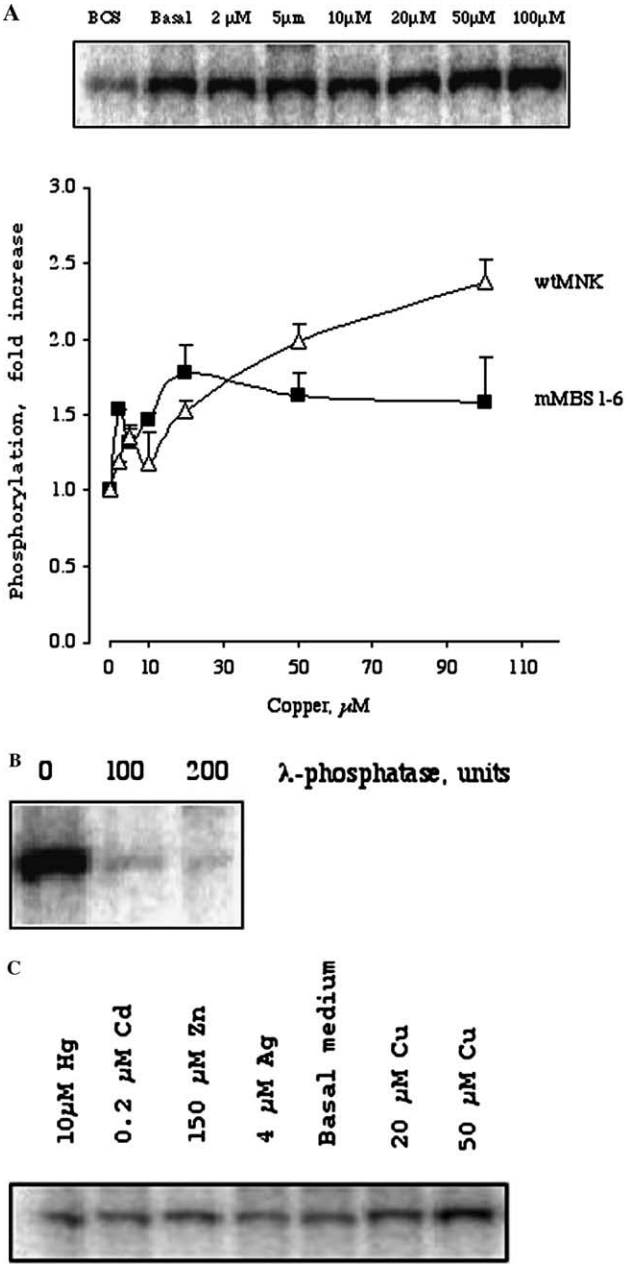

Fig. 2. Metabolic labelling of wtMNK and mMBS overexpressed in cultured Chinese hamster ovary cells. (A) A typical autoradiogram of metabolically labelled, with $^{32}P_i$, wtMNK for 3 h (see Materials and methods for details). The graph shows the copper concentration-dependent labelling of wtMNK and mMBS; the level of phosphorylation was related to phosphorylation in basal media. The data are shown as means ± SE ($n = 3$). (B) De-phosphorylation of wtMNK with λ-phosphatase. (C) Copper-specific phosphorylation of wtMNK. The cells were metabolically labelled, with $^{32}P_i$, in the presence of indicated concentrations of heavy metals for 3 h, $HgCl_2$, $CdSO_4$, $ZnSO_4$, and $AgNO_3$, and processed as described above and in Materials and methods.

More than 95% of the phosphorylated wtMNK intermediate was hydrolysed by λ-phosphatase supporting the protein kinase-dependent nature of the phosphorylation

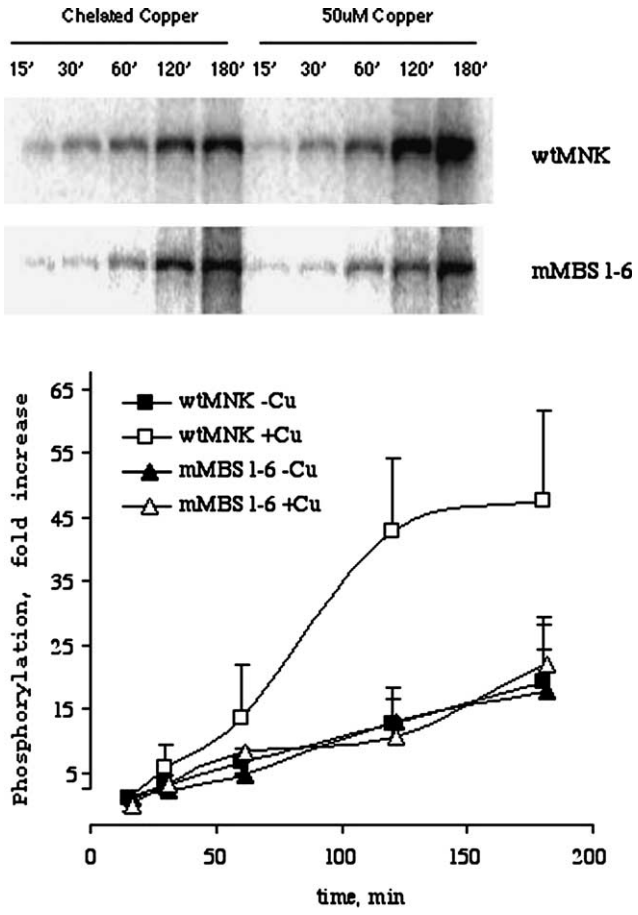

Fig. 3. Copper-dependent and copper independent time-course of phosphorylation of wtMNK and the mMBS MNK mutant. The photographs represent typical autoradiograms of a time-course experiment. The diagram represents the comparative rates of phosphorylation of wtMNK and the mMBS mutant. Error bars are removed for clarity ($n = 2$–3). Fold increase is in comparison to 15 min metabolic labelling (see Materials and methods for details).

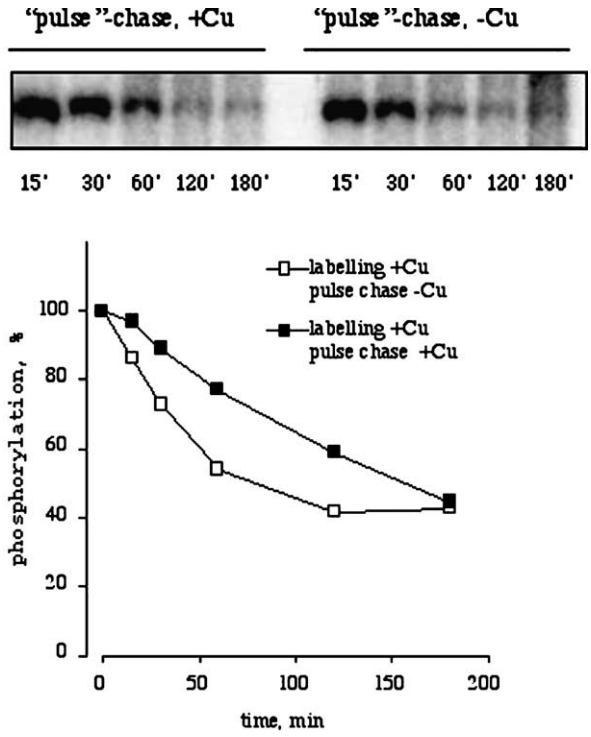

Fig. 4. "Pulse-chase" of metabolically labelled wtMNK. The cells were incubated with $^{32}P_i$ for 3 h in the presence of 50 μM copper, after which the phosphate-free medium was replaced with normal, phosphate-complemented culture media, for indicated periods of time. The "chase" was conducted in the absence of elevated copper or in the presence of 50 μM copper.

(Fig. 2B). Importantly, the phosphorylation was copper-specific as other heavy metals, such as Hg, Zn, Cd, or Ag, had no detectable effect on the level of the MNK labelling (Fig. 2C). Finally, copper-induced phosphorylation was not related to de novo protein synthesis in the presence of copper, as no detectable increase in MNK turn-over or synthesis was observed during the course of experiments (data not shown).

Examination of the time-course of the phosphorylation of wild-type and mutant MNK in the absence or presence of 50 μM copper indicated similar basal (no added copper) rates of phosphorylation. Consistent with the results presented in Fig. 2, copper stimulated the rate of phosphorylation of wild-type MNK with no activation of the phosphorylation of mMBS above basal rates (Fig. 3).

The dynamics of MNK phosphorylation were further investigated in a "chase" experiment, where $^{32}P_i$ in the media was replaced by "cold" P_i (Fig. 4). Interestingly, the phospho-MNK turned over more slowly in the presence of copper than under basal copper concentrations (half life = 160 min compared with 90 min).

In order to examine the copper-dependent phosphorylation of MNK in more detail, the phospho-MNK was subjected to 2-dimensional (2-D) tryptic phosphopeptide mapping. Wild-type MNK was basally phosphorylated on three major phosphopeptides (labelled 1–3 in Fig. 5 as well as on minor peptides (labelled 4 and 5 in Fig. 5). Addition of 100 μM copper resulted in specific phosphorylation on a novel phosphopeptide labelled "6," as well as an increased phosphorylation of phosphopeptide 1 (Fig. 5A). The non-trafficking mutant mMBS was also phosphorylated on the constitutive sites (Fig. 5A). However, while some copper-dependent phosphorylation of peptide 1 was observed, phosphorylation of phosphopeptide "6" was not detected (Fig. 5A lower panels). Phosphoamino acid analysis of extractable phosphopeptides revealed that all were phosphorylated on serine residues (Fig. 5B).

Discussion

Protein kinase-dependent phosphorylation is important in regulating both the catalytic activity and subcellular localisation of various ATPases, including the

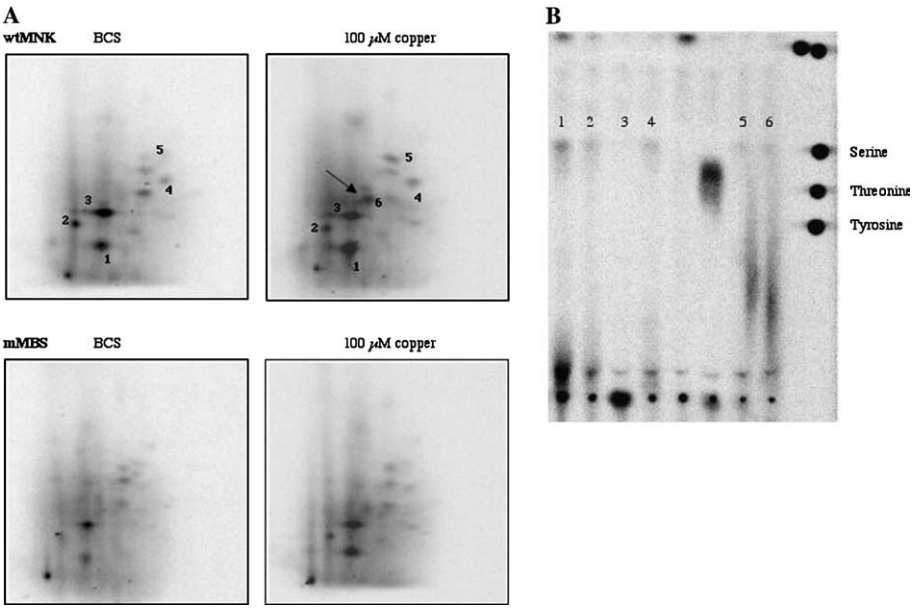

Fig. 5. The 2-dimensional tryptic phosphopeptide map of wtMNK and the mMBS MNK mutant treated with the copper chelator BCS or 100 μM copper. The cells were metabolically labelled with $^{32}P_i$ following their incubation in the phosphate-free medium and processed as described in Materials and methods. (A) Approximately equal amounts of radioactivity were loaded on TLC plates. Peptides labelled as 1–6 are those used for phosphoamino acid analysis. The arrow indicates a novel phosphopeptide (''6''), which is not observed in the BCS-treated wtMNK or in the mMBS cells. (B) The phosphoamino acid analysis of wtMNK (see Materials and methods) indicates all peptides with detectable phosphoamino acid release being serines (numbered 1–6).

Na/K-ATPase, Ca-ATPases, and CFTR [7–9]. Copper-dependent phosphorylation of the liver-specific copper P-type ATPase, WND, has been reported [10]. It was suggested that phosphorylation may be linked to trafficking of WND, although no causal relationship was demonstrated [10]. Our current study provides the first evidence that MNK is phosphorylated by an upstream protein kinase targeting serine residues. Our data provide the first insight into the dynamics of copper-dependent phosphorylation of MNK and also suggest an important role of N-terminal high affinity copper-binding sites in this process.

Our primary aim was to establish whether MNK could undergo reversible copper-induced phosphorylation indicative of a role for post translational modulation of MNK activity and/or localisation. We found that in the presence of basal copper concentrations (<1 μM), the level of phosphorylation was significantly up-regulated compared to copper-depleted conditions, thus indicating that MNK phosphorylation was extremely sensitive to the modulation of even such low copper levels. The phosphorylation was further increased in the presence of elevated copper concentrations (Fig. 2), which coincided with relocalisation of MNK from the TGN to the PM [3].

Furthermore, the wtMNK protein was constitutively phosphorylated on serine residues in three major phosphopeptides, in the presence of copper chelators. This basal phosphorylation may play a ''housekeeping role,'' for example, in determining TGN localisation of MNK under basal copper conditions and/or stabilising the conformation of the protein. The 2-D tryptic phosphopeptide map revealed two copper-stimulated phosphopeptides (Fig. 5). While phosphopeptide ''1'' was also phosphorylated in the basal state, phosphopeptide ''6'' appeared to be phosphorylated in the presence of copper and this phosphorylation was not observed in the case of a non-trafficking mutant. Taken together these observations imply phosphorylation at this site may play a critical role in regulating MNK function in response to increases in copper concentration. Whether this is due to the action of a copper-activated protein kinase, copper-dependent trafficking to allow association with an active protein kinase, and/or to copper-dependent structural changes in the MNK protein allowing subsequent phosphorylation is unclear.

Our initial in vitro experiments demonstrated that purified membrane vesicles could phosphorylate MNK in a copper-dependent fashion, consistent with the hypothesis that the in vivo phosphorylation is mediated via a copper-dependent protein kinase. Indeed copper deficiency and/or copper overload have been shown to alter the activity and expression of protein kinases including the PI3K/Akt and MAPK pathways [14,15]. Importantly, ''pulse''-chase experiments revealed that the stability of the phosphorylated form of MNK was also increased in the presence of copper (Fig. 4). This observation raises the possibility that copper may also inhibit the dephosphorylation of MNK, effectively amplifying its effects on MNK phosphorylation. Indeed, copper has been observed to inhibit protein phosphatase activity in

some cell systems [16,17]. We have previously demonstrated exocytic trafficking of MNK to the PM in the presence of elevated copper concentrations, and the return of MNK to the TGN when copper concentration subsides to the basal level [3]. The return of cells to basal copper may relieve the inhibition of this putative phosphatase allowing de-phosphorylation of MNK and return to the basal steady-state localisation of MNK at the TGN.

The N-terminal putative high affinity metal-binding sites (MBS) have been shown to selectively bind copper [18] and regulate the catalytic function of MNK [11]. In addition, the mutation of all six MBSs prevented the mutant MNK from copper-induced exocytic trafficking to the PM [5]. Here we observed a substantial difference in copper-dependent phosphorylation of the wild-type MNK and the mutant MNK with all MBSs site-specifically mutated. While the basal phosphorylation of the mutant protein remained unaffected by the mutations, copper-dependent phosphorylation was inhibited, and in particular, phosphorylation of the novel peptide "6" was abolished. The basis for the lack of copper dependence of phosphorylation of the mMBS mutant remains to be elucidated. It is possible that the inability of the mutant protein to bind copper results in a conformation that is inaccessible for phosphorylation. Conversely, the inability of this mutant to traffic to the PM may preclude its phosphorylation by the PM associated protein kinase. Recent studies using WND also suggested the importance of the N-terminal copper-binding sites in kinase-dependent phosphorylation. This was based on the observation that the basal phosphorylation of the mutant form of WND, which lacked the whole N-terminus and first four transmembrane domains, could not be up-regulated in the presence of elevated copper [10].

The lack of significant copper-dependent phosphorylation of mMBS compared to significant effects observed in wtMNK highlights a potential importance of phosphorylation in such copper-specific functions of MNK as copper-induced trafficking and catalysis. We predict that the kinase-dependent phosphorylation of phosphopeptide "6" may play an important role in the regulation of physiological function of MNK. Further studies involving the identification of this peptide and structure function analysis of this site are required to assess the role of phosphorylation in regulating the physiological functions of MNK.

Acknowledgments

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