Copper depletion down-regulates expression of Alzheimer’s disease Amyloid-β precursor protein gene

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SUMMARY

Alzheimer’s disease is characterised by the accumulation of amyloid-β peptide, which is cleaved from the amyloid-β precursor protein (APP). Reduction in levels of the potentially toxic amyloid-β has emerged as one of the most important therapeutic goals in Alzheimer’s disease. Key targets for this goal are factors that affect the regulation of the APP gene. Recent in vivo and in vitro studies have illustrated the importance of copper in Alzheimer’s disease neuropathogenesis and suggested a role for APP and amyloid-β in copper homeostasis. We hypothesised that metals and in particular copper might alter APP gene expression. To test the hypothesis, we utilised human fibroblasts over-expressing the Menkes protein (MNK), a major mammalian copper efflux protein. MNK deletion fibroblasts have high intracellular copper, while MNK over-expressing fibroblasts have severely depleted intracellular copper. We demonstrate that copper depletion significantly reduced APP protein levels and down-regulated APP gene expression. Furthermore, APP promoter deletion constructs identified the copper-regulatory region between –490 to +104 of the APP gene promoter in both basal MNK over-expressing cells and in copper-chelated MNK deletion cells. Overall these data support the hypothesis that copper can regulate APP expression and further support a role for APP to function in copper homeostasis. Copper-regulated APP expression may also provide a potential therapeutic target in Alzheimer’s disease.
INTRODUCTION

A number of neurodegenerative disorders including Alzheimer’s disease (AD), Parkinson’s disease, Amyotrophic lateral sclerosis and Prion disease have been closely linked to disturbances in copper homeostasis in the central nervous system and the brain (1,2). AD is the most common progressive neurodegenerative disorder in elderly people and is characterised by neuronal loss with the accumulation of senile plaques and neurofibrillary tangles. The major proteinaceous component of senile plaques is a 39-42 amino acid peptide, termed Amyloid-β peptide (Aβ) (3), that is proteolytically cleaved from the larger amyloid-β precursor protein (APP) (4).

An apparent relationship between copper homeostasis, APP and AD has been suggested (1). APP has a specific type II copper-binding site that binds copper with a Kd of 10 µM (5) and can modulate copper-induced toxicity and oxidative stress in primary mouse neuronal cultures (6). APP knockout mice have increased copper levels in the brain (7) while conversely, transgenic over-expression of APP and Aβ in mice correlates with reduced brain copper levels (8). In a cellular system over-expressing APP, increasing copper concentration can modulate APP processing, stimulating levels of cell-bound and secreted forms of APP with reduced production of Aβ (9). The importance of copper in AD pathology has also been demonstrated by the ability of Aβ to bind copper with a high affinity (10) promoting amyloid plaque aggregation and neurotoxicity (11). Conversely, treatment with a copper-zinc

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1 Alzheimer’s disease (AD), Amyloid-β-peptide (Aβ), Amyloid-β precursor protein (APP), Menkes protein (MNK), Metallothionein (MT), Copper response element (CuRE), Metal response element (MRE), ACE1/AMT1 response element (Ace/AmtRE), MRE-like sequence (MLS), ACE1/AMT1-like sequence (ALS), Chloramphenicol acetyl transferase (CAT), β-Galactosidase (β-Gal)
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A chelator can disaggregate Aβ both in vitro and in transgenic mouse models in vivo (12).

The APP gene is expressed in all major tissues but predominantly in the brain, where expression is primarily in neurons (13). Although there is both developmental and cell-type specific regulation of APP, expression in the adult is mostly ubiquitous (14). The regulation of the APP gene as a pathogenic factor for AD has received considerable attention. Down syndrome patients, who have an extra copy of the APP gene, invariably exhibit early onset AD-like pathology (15). Over-expression of APP in certain areas of the brain in AD patients also suggest that the regulation of APP might be an important factor in the neuropathology of AD (16). These observations illustrate the importance of elucidating mechanisms of APP gene regulation in the development of AD.

The human APP promoter closely resembles that of a typical housekeeping gene and contains the consensus sequences for the binding of several transcription factors (17,18). Nerve growth factor, interleukin-1, retinoic acid and various transcription factors are among several cellular mediators that cause an increase in APP mRNA levels in neuronal and non-neuronal cells (19). In contrast, thyroid hormone and interferon-gamma have been reported to down-regulate APP expression (20,21). Recently, an iron responsive element located in the 5’-untranslated region has been implicated in the regulation of APP expression (22). We hypothesised that metals and in particular copper might alter APP gene expression.

To investigate the role of copper in APP gene regulation, we utilised a novel approach involving cultured human fibroblasts over-expressing the Menkes protein (MNK; encoded by ATP7A), a major mammalian copper translocating P-type ATPase involved in copper efflux (23-25). Cells lacking the MNK protein show high
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intracellular copper levels due to the lack of active copper efflux, while cells transfected and over-expressing MNK have markedly reduced copper levels (25).

We report that depletion of intracellular copper results in significant reduction of APP gene expression. In addition, APP promoter analysis suggests putative metal regulatory elements may be involved in mediating the response to copper depletion to regulate APP gene expression. Overall, these data suggest that copper is a required co-factor for the basal regulation of the APP gene and supports the increasing evidence that APP is involved in copper homeostasis as a copper detoxification/efflux protein.

EXPERIMENTAL PROCEDURES

Cell lines — Human skin fibroblast cells, Me32a, were isolated from a classical Menkes disease patient (26). The patient carried a 4 bp deletion that resulted in a frame-shift mutation and premature stop codon of the MNK gene, whose expression was undetectable by western and northern analysis (25-27). Me32a cells were immortalised by SV40 gene transfer to derive Me32aT22/2L and designated MNK deletion (25). The 4.6 kb cDNA encoding the human MNK protein was cloned into a mammalian expression vector and transfected into Me32aT22/2L (25). Two independently derived clones that expressed MNK were isolated, MNK A12-H9 and MNK C3-C1, and designated MNK transfected A and MNK transfected B (25). The empty mammalian expression vector, pCMB77, was transfected alone into MNK deletion to derive Me32aT22/2L(pCMB77) and designated vector only (25). The SV40-immortalised human fibroblast cell line, normal human (GM2069), and human neuroblastoma cell line, human neuron (SY5Y), were used as controls.

Culture conditions — Cell lines, MNK deletion and normal human were maintained in basal medium, which consisted of Eagle’s basal media (BME; Thermo Trace, Australia) (24). The BME was supplemented with 10% foetal calf serum (FCS; Thermo Trace, Australia), 2 mM L-glutamine, 0.2mM proline, 20 mM HEPES
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*(N*-2-hydroxy-ethyl-piperazine-*(N’*-2-ethylsulphonic acid) and 0.2% (w/v) sodium bicarbonate. *Vector only*, MNK transfected A and MNK transfected B cell lines were maintained in 10% BME as above with the additional supplement of 400 µg/ml Geneticin (Invitrogen). The cell line, human neuron, was maintained in basal medium, which consisted of RPMI 1640 (Thermo Trace, Australia). The RPMI 1640 was supplemented with 20% FCS, 0.8 mM L-glutamine, 0.4 mM sodium pyruvate, 0.02 mM uridine, 16 mM HEPES and 0.05% (w/v) sodium bicarbonate. The basal copper concentration of 10% BME and 20% RPMI 1640 was 0.78 µM (0.05 µg/ml) and 1.02 µM (0.07 µg/ml), respectively as measured by atomic absorption spectroscopy using a Perkin Elmer 5000 Atomic Absorption Spectrophotometer. Cells were incubated at 37°C.

*Bioinformatics* — The human APP gene promoter sequence (GenBank Accession Number D87875) and rhesus monkey APP gene promoter sequence (GenBank Accession Number AF067971) was searched for copper response elements (28) (CuRE consensus sequence 5’ WWWWTTTGCKCR 3’), ACE1/AMT1 response elements (29) (Ace/AmtRE consensus sequence 5’ THNNGCTG 3’) and metal response elements (30) (MRE consensus sequence 5’ TGRCRCNC 3’) consensus sequences using TESS: Transcription Element Search Software (31). In addition, promoters were searched for ACE1/AMT1-like sequences (ALS), with no more than one base mismatch to the first two ACE1/AMT1 consensus residues (5’ THNNGCTG 3’); and MRE-like sequences (MLS), with no more than one base mismatch from the last three MRE consensus residues (5’ TGRCRCNC 3’).

*Antibodies* — Anti-MNK polyclonal antibodies raised to the MNK N-terminal region (24) were diluted 1:2500 for use in western blot analysis. Anti-APP (WO2) monoclonal antibodies raised to the Amyloid-β region (32) were diluted 1:1000 for use in immunofluorescence studies and 1:10000 for use in western blot analysis.
Steady-state measurement of $^{64}Cu$ accumulation — Cells were grown to near confluency in basal growth medium. The medium was replaced with BME medium supplemented with 2% FCS containing 5-10 µCi/ml $^{64}Cu$ (as CuCl$_2$; ARI, Lucas Heights, NSW, Australia). After incubation at 37°C for 16, 24 and 36 hours, cells were immediately harvested to obtain total $^{64}Cu$ accumulated at the end of the time period (four 35 mm petri-dishes containing 1X $10^6$ cells per time point). All cells were washed twice in ice-cold serum-free medium to remove any non-specifically bound $^{64}Cu$ and harvested by dissolution in 1.5 % SDS, 2 mM EDTA. Cell lysates were scraped and collected. $^{64}Cu$ was measured in cell lysates using an LKB-Wallac Ultragamma counter. Protein concentration was estimated in each cell lysate using a Bio-Rad Protein Assay (33).

Total cellular measurement of copper, zinc and iron — Cells were grown for 4 days in basal growth medium as described above. Cells were harvested as previously described (34) and copper, zinc and iron were analysed using a Perkin Elmer 5000 Atomic Absorption Spectrophotometer.

Indirect immunofluorescence — Cells were seeded in basal medium onto 13mm round cover-slips 48 hours prior to immunofluorescence. Cover-slips were washed in ice-cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 15 min, washed several times with PBS and permeabilised with 0.1% Triton X-100 in PBS for 5 min, washed several times in PBS then blocked overnight in PBS containing 1% bovine serum albumin (BSA). All antibody incubations were for 1 hour in 1% BSA/PBS. Monoclonal APP antiserum was visualised using Alexa-488™-conjugated to goat anti-mouse IgG antibodies (Molecular Probes). Cover-slips were washed overnight in PBS then mounted using 100 mg/ml DABCO (Sigma) in 90% glycerol. Confocal microscopy was carried out using an Olympus BX60 microscope with a 60x PlanApo lens with a 1.40 N/A. This microscope was
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Protein extraction and Western immunoblot analysis — Cell lines were sonicated in 62.5 mM Tris-HCl pH 6.8 buffer containing 2% SDS, 10 mM DTT, 1 mM EDTA and COMPLETE™ EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals). 50 µg of protein extracts were fractionated on NuPage™ Bis-Tris (4-12%) gradient acrylamide gel (Invitrogen) and electroblotted overnight to nitrocellulose filters. Detection of MNK and APP protein was performed using an “ECL+Plus” chemiluminescence kit (Amersham Pharmacia Biotech), according to the manufacturer’s instructions. Protein concentrations were measured using a Bio-Rad Protein Assay (33), and MultiMark® (Invitrogen) protein molecular weight standards were loaded. Purified human APP695 isoform ectodomain was loaded as a control (35).

Total RNA extraction and Northern blot analysis — Total RNA was extracted from cell lines using the Total RNAeasy kit (QIAGEN). 5µg total RNA samples were denatured in formamide, formaldehyde, MOPS and ethidium bromide at 65°C for 15 min, cooled on ice, and electrophoresed on a 1.0% agarose-formaldehyde gel. The gel was blotted on Hybond-N+ nitrocellulose filter and immobilised by 50 mM sodium hydroxide. Each filter was pre-hybridised in hybridisation buffer containing 50 X Denhardt’s reagent, sheared Salmon sperm DNA, 0.5 M EDTA, 1 M Tris, 20% SDS and 1 M NaPO₄ for at least 2 hours. The filter was hybridised with a α-³²P-dATP labelled cDNA probe using the Prime-a-Gene labelling System (Promega). After hybridisation, the filters were washed to a stringency of 0.01% SDS, 0.1 X SSC at 60°C. APP cDNA probe corresponded to 3kb fragment generated from human APP cDNA. Equal loading of samples was verified by re-hybridising the filter with the “housekeeping gene cDNA probe” rat glyceraldehyde phosphate dehydrogenase.
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Preparation of plasmid constructs — The promoter constructs pRSV-βGal, pBLCAT3, pβBIV, pβPB and pβHB as previously described (36,37). Plasmids were prepared for transfection using an EndoFree® plasmid maxi kit (QIAGEN) according to the manufacture’s instructions.

Transient transfection and lysate preparation — Cells were seeded in 6-well plates 24 hours prior to transfection in basal medium. All cells were transfected with FuGENE 6 (Roche Molecular Biochemicals) according to manufacturer’s instructions. Transfection mixture was prepared containing 0.5µg pRSV-βGal and either 3µg of pBLCAT3, pβBIV, pβPB or pβHB per well. Prior to transfection, medium was changed to basal medium (0.78 µM Cu), copper-supplemented medium (50, 100, 250 or 500 µM Cu) or copper-chelator diamsar (38) treated medium (5, 10 or 50 µM). 48 hours post-transfection cells were washed with ice-cold PBS several times, lysed in Reporter Gene Lysis Buffer (Roche Molecular Biochemicals) and collected by microfuge centrifugation (16,000 g) to remove any cellular debris. Protein concentration was measured in each cell extract using a Bio-Rad Protein Assay (33).

β-Galactosidase (β-Gal) assay — β-Gal activity was used to standardise transfection efficiency for subsequent CAT ELISA assays. The cell extracts from transfected cells were analysed for their β-Gal activity using a β-Gal Enzyme Assay System according to manufacturer’s instructions (Promega). Briefly, 5µg of protein (an amount within the linear range of the assay) was assayed for 30 min at 37°C in 2X Assay Buffer before the reaction was stopped with the addition of 1 M sodium
Running Title: Copper depletion down-regulates APP gene expression carbonate. The absorbance was measured on a Bio-Rad 2550 EIA plate reader at 420 nm.

*Chloramphenicol acetyl transferase (CAT) promoter assay* — The cell extracts from transfected cells were analysed for their CAT activity using a colorimetric ELISA assay according to manufacturer’s instructions (Roche Molecular Biochemicals). Briefly, 50µg of protein (an amount within the linear range of the assay) was placed on anti-CAT coated microtiter plate modules and allowed to bind for 1 hour at 37°C. The plates were washed thoroughly after each step. Next, a digoxigenin-labelled anti-CAT antibody was added to the samples and incubated for 1 hour at 37°C. A subsequent antibody, anti-digoxigenin conjugated to peroxidase, was placed in the wells for 1 hour at 37°C. Finally, peroxidase substrate, ABTS (Roche Molecular Biochemicals), was added and the absorbance measured at 405 nM using an ELISA plate reader. CAT activity was determined from the ratio of pg CAT/milli unit β-Gal per µg protein.

*Statistical analysis* — One-way ANOVA of more than two means followed by Bonferroni’s multiple comparison of mean’s post test was performed for northern analysis, $^{64}$Cu accumulation, cellular metal determination, and promoter assays using Graphpad Prism3 for Macintosh (GraphPad Software Inc.). Statistically significant was defined as $P < 0.05$.

**RESULTS**

(Table I suggested location).

*Intracellular copper levels, but not zinc or iron, are severely decreased in MNK transfected fibroblasts* — Immortalised human fibroblasts isolated from a Menkes disease patient (Table 1), herein referred to as MNK deletion cells and MNK deletion cells stably transfected with and hence over-expressing the MNK efflux protein (Table 1), herein referred to as MNK transfected cells, represented powerful tools to
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investigate the hypothesis that copper levels can modulate APP gene expression. This is due to MNK deletion cells having high intracellular copper, while MNK transfected cells have low intracellular copper (25). To confirm that MNK deletion and MNK transfected fibroblast cell lines (Fig. 1A) have dramatically altered copper levels, we investigated cells for intracellular levels of copper as well as zinc and iron. (Figure 1 suggested location).

Intracellular copper levels were measured using two independent approaches. The first was to analyse cells for copper accumulation using the Cu radioisotope, $\text{^{64}Cu}$, under steady state conditions. $\text{^{64}Cu}$ analysis showed MNK deletion and vector only control cells maintained copper levels significantly higher than normal human fibroblast cells with an average increase of 125% (Fig. 1B). MNK transfected cells maintained copper levels significantly lower than normal human fibroblast cells with an average decrease of 82% (Fig. 1B). MNK transfected cells also maintained copper levels significantly lower than MNK deletion cells with an average decrease of 92% (Fig. 1B).

The second method of analysis examined the total amount of copper present in cell pellets utilising atomic absorption spectroscopy. This analysis showed MNK deletion and vector only control cells contained significantly elevated copper levels compared to normal human fibroblast cells with an average increase of 65% (Fig. 1C). MNK transfected cells contained significantly reduced copper levels, with an average decrease of 79%, compared to normal human fibroblast cells (Fig. 1C). MNK transfected cells also contained significantly reduced copper levels compared to MNK deletion cells with an average decrease of 87% (Fig. 1C). There was no significant difference in the zinc and iron content of these fibroblast cell lines (Fig. 1D-E).

Overall, the cellular metal analysis demonstrated that by over-expression of MNK in a MNK deletion background, we could dramatically alter intracellular copper.
Running Title: Copper depletion down-regulates APP gene expression levels, providing an ideal cellular system to test the hypothesis that copper can regulate the expression of the APP gene.

(Figure 2 suggested location).

*MNK transfected fibroblasts have decreased APP protein and APP mRNA levels* — *MNK deletion* and *MNK transfected* cell lines were analysed for APP protein expression using the WO2 monoclonal antibody (32). WO2 is raised to the Aβ region of APP and as such can detect both APP and Aβ protein species. Immunofluorescence analysis showed *MNK deletion, vector only control* and *normal human* fibroblasts all had APP and/or Aβ levels similar to that observed in control human neurons (Fig. 2A). Interestingly, APP and/or Aβ protein was not detectable via immunofluorescence in *MNK transfected* cells (Fig. 2A).

APP protein expression in fibroblast lines was further examined using western blot analysis. APP protein isoforms were detected as bands between 90 and 110 kDa (Fig. 2B). *MNK deletion, vector only control* and *normal human* fibroblasts produced protein bands that were comparable to control human neurons (Fig. 2B). However, in *MNK transfected* cells, the protein bands detected were severely decreased in their intensity when compared to *vector only control, normal human* fibroblasts and control human neurons (Fig. 2B). Possible mechanisms for the reduced levels of APP protein in *MNK transfected* cell lines could involve effects at the transcriptional, translational or post-translational levels.

We performed northern analysis of *APP* mRNA levels in these fibroblast lines. A single *APP* mRNA transcript band was observed in *MNK deletion, vector only control* and *normal human* fibroblast cell lines that corresponded to the transcript detected in control human neurons (Fig. 2C, upper panel). *MNK transfected* cells also had a single *APP* mRNA transcript. However, the observed band intensity was severely reduced compared to control lines and to the control housekeeping transcript.
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GAPDH (Fig. 2C, lower panel). Quantification of APP mRNA levels by densitometry showed no significant difference in APP expression between MNK deletion, vector only control, normal human fibroblast and human neuron cell lines (Fig. 2D). Notably, MNK transfected cells showed a significant decrease in APP expression with a down-regulation of approximately 83% compared to MNK deletion cells (Fig. 2D). These data provide strong support for the hypothesis that reduced copper levels result in down-regulation of APP gene expression in MNK transfected cultured human fibroblasts.

(Table II suggested location).

The human APP and rhesus monkey APP gene promoters contain a number of putative metal regulatory sequences — The human APP gene promoter (GenBank D87875) and rhesus monkey APP gene promoter sequences (GenBank AF067971) were searched for copper response elements (CuRE) found in yeast copper uptake genes FRE1, CTR1 and CTR3 promoters (28); ACE1/AMT1 metal response elements found in yeast copper detoxification genes CUP1, CRS5 and SOD1 promoters (29); and metal response elements (MRE) found in the mammalian copper detoxification gene metallothionein (MT) promoter (30). In addition promoters were searched for ACE1/AMT1-like sequences and MRE-like sequences. Utilising this search criteria, a number of putative copper and metal response elements were identified in the human and rhesus monkey APP promoters (Table 2). The contribution of these elements to a possible copper-responsive regulation of the APP gene has not yet been determined.

(Figure 3 suggested location).

Copper depletion decreases APP gene expression through the 5′-APP promoter — To investigate the role of putative metal response element(s) in the down-regulation of the APP gene, promoter analysis was performed with regions of the 5′-
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APP promoter fused to a chloramphenicol acetyl transferase (CAT) reporter gene (Fig. 3A). The promoter constructs used here have all been characterised functionally and all displayed a significant level of promoter activity in neuronal PC12 cells (36,37). To test whether these reporter constructs are regulated by intracellular copper levels, MNK deletion, vector only control and MNK transfected cells were transiently transfected in basal media for 48 hours with basic promoter-less CAT, -3416 to +104 5’-APP-CAT, -1131 to +104 5’-APP-CAT and -490 to +104 5’-APP-CAT promoter constructs. MNK transfected cell lines demonstrated a significant decrease in APP promoter activity with the –3416 to +104, –1131 to +104 and –490 to +104 APP promoter fusion constructs when compared to MNK deletion cells (Fig. 3B). The promoter activity of MNK transfected cell lines was down-regulated by 75% on average when compared to the promoter activity observed for MNK deletion and vector only control cell lines (Fig. 3B).

To further clarify the response of these putative regulatory element(s), promoter analysis was performed under copper-supplemented and copper-chelation conditions. MNK transfected cells were chosen for further analysis under copper-supplemented conditions to determine if the down-regulation of the APP promoter could be restored. MNK transfected cells were transiently transfected with promoter constructs and grown in basal and copper-supplemented medium for 48 hours. There was no significant difference in APP promoter activity observed between cells grown in basal media and those grown in copper-supplemented media for MNK transfected cells (Fig. 3C). Although in copper-supplemented media, copper levels are increased beyond those observed for MNK deletion cells grown in basal media (Table 3), it is possible that the distribution of copper within intracellular pools in copper-supplemented MNK transfected cells may be altered.
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To confirm that the differential promoter response seen was due to low intracellular copper levels, we depleted copper levels in MNK deletion cells using the copper chelator, diamsar (38). MNK deletion cells were transiently transfected with promoter constructs and grown in basal media and media containing the copper chelator, diamsar for 48 hours. Analysis showed a significant difference between non-chelated and chelated cells with the –3416 to +104, –1131 to +104 and –490 to +104 APP promoter fusion constructs (Fig. 3D). Chelation with 5, 10 and 50µM diamsar was sufficient to down-regulate APP promoter activity by 53, 73 and 80% respectively (Fig. 3D).

Overall analysis of APP promoter activity provided strong support for the hypothesis that reduced copper levels result in down-regulation of APP gene expression in cultured human fibroblasts. Furthermore, investigations utilising APP promoter deletion constructs narrowed the copper-responsive regulatory region to between –490 to +104 of the APP gene promoter in both basal MNK transfected cells and in copper-chelated MNK deletion cells (Fig. 3).

DISCUSSION

Increasing evidence has implicated APP and Aβ in copper homeostasis (8,39). Since it has been demonstrated in various systems that genes involved in copper homeostasis can be regulated by cellular copper levels (28-30), as evidenced by the yeast copper uptake genes FRE1, CTR1 and CTR3 (28); yeast copper detoxification genes CUP1, CRS5 and SOD1 (29) and the mammalian copper detoxification gene MT (30), we hypothesised that copper may also play a role in the regulation of the APP gene. To investigate the role of copper in APP gene regulation, we utilised a system where intracellular copper levels could be genetically manipulated through altered expression of the MNK copper efflux protein (Table 1). Cells lacking the MNK protein show high intracellular copper levels due to reduced copper efflux (25).
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Restoration of MNK function by over-expression of MNK in a MNK deletion background restores copper efflux ability, resulting in dramatically decreased intracellular copper levels (25). This system leads to a marked reduction of intracellular copper levels via over-expression of MNK, in a MNK deletion background (25).

Here we report for the first time, evidence that decreased copper can down-regulate expression of the human APP gene. This demonstrates a previously uncharacterised aspect of regulation of the human APP gene and further supports a role for the APP protein in copper homeostasis. This study also suggests that copper-factor(s) in the region −490 to +104 of the APP promoter are required for regulation of the APP gene.

The effect of decreased copper on APP gene regulation was observed in two independently derived MNK transfected fibroblast cell lines that constitutively express high levels of MNK. The use of two independent methods for determining the steady-state levels of copper in these fibroblast cell lines confirms that in MNK transfected cell lines the cellular copper levels are reduced by about 90% compared to the parental MNK deletion cell line (Fig. 1). These results are consistent with previous published copper data (25). Importantly, the levels of zinc, a known transcriptional co-factor, and iron, recently reported to be involved in regulating the 5’ UTR of the APP gene (22), were unaffected (Fig. 1). The reduction of copper levels in MNK transfected cells correlates with a decrease in APP protein levels and on average an 83% down-regulation of APP gene expression detected in MNK transfected fibroblasts (Fig. 2). Elevated copper levels observed in MNK deletion and vector only control cells relative to normal human fibroblasts (Fig. 1), were not associated with altered APP expression (Fig. 2). These results suggest that copper-
Running Title: Copper depletion down-regulates APP gene expression related down-regulation of APP gene expression is associated with the very low copper levels attained in MNK transfected fibroblast cell lines.

To evaluate the role of the APP gene promoter in the copper related down-regulation of the APP gene we utilised APP promoter deletion constructs fused to a CAT reporter system. Analysis was performed under basal medium conditions, where it was predicted that MNK transfected cell lines would have decreased promoter activity compared to MNK deletion cells due to reduced copper levels (Fig. 1). MNK transfected cell lines demonstrated a significant decrease in basal APP promoter activity when compared to MNK deletion cells for all promoter deletion constructs analysed (Fig. 3B). MNK transfected cell lines are down-regulated by 75% on average when compared to promoter activity observed for MNK deletion and vector only control cell lines (Fig. 3B). This result is consistent with the approximate 83% down-regulation of APP mRNA expression observed from northern analysis.

Whilst copper supplementation of MNK transfected cells was shown to increase cellular copper levels (Table 3), APP promoter activity was not restored (Fig. 3C). This may result from a lack of available copper in the pool responsible for activating APP promoter expression, and could be due to sequestration of copper via MNK in intracellular compartments or vesicles in cells over-expressing MNK (40,41).

Under copper-depleted conditions in MNK deletion cells, the APP promoter activity was significantly reduced by up to 80% using the Cu$^{2+}$ “cage” chelator diamsar (Fig. 3D). MNK deletion cells in the presence of 10µM and 50µM diamsar copper chelator showed a similar down-regulation of the APP promoter to that observed under basal medium conditions for MNK transfected cells (Compare Fig. 3B to Fig. 3D). This suggests that the putative regulatory element(s), responsible for the down-regulation of the APP gene observed in MNK transfected fibroblasts, are located within the region –490 to +104 of the APP promoter.
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It has recently been suggested that both APP and Aβ can function in copper efflux/detoxification (8,39). This was based on: i) the structural homology of the APP copper binding-domain to known copper chaperone and copper efflux protein copper-binding domains (39); ii) the extracellular localisation of the copper binding domain (4); iii) the findings that APP knockout mice show increases in brain copper levels (7), whilst transgenic overexpression of APP and Aβ results in reduced brain copper levels (8); iv) the observation that elevated copper levels results in a decrease in the amyloidogenic pathway and stimulation of the non-amyloidogenic pathway of APP cleavage, thus releasing the secreted APP ectodomain with concomitant efflux of copper (9).

The regulation of APP gene expression by copper described in the current studies also strongly supports a role for APP in copper efflux/detoxification. In MNK deletion cells the APP protein may have a significant role in copper detoxification/efflux, due to the absence of the MNK protein. When MNK deletion cells are placed under conditions of copper depletion, either by the over-expression of transfected wild-type MNK protein or copper chelation, the requirement for copper detoxification/efflux is presumably diminished, resulting in the observed down-regulation of the APP gene in order to maintain normal copper homeostasis. Also the significant down-regulation of APP gene expression observed under low copper conditions is similar to known transcriptional regulation mechanisms for copper detoxification/efflux genes in yeast and humans (29,30). Given the non-amyloidogenic processing of APP and in vivo reduction of Aβ under elevated copper conditions (9,42,43), it is possible that down-regulation of the APP gene under low copper conditions acts as a preventative measure to decrease the production of Aβ and effectively guard against amyloidogenic processing of APP.
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In summary, utilising a novel cellular copper efflux system, our results strongly suggest that at least in human fibroblasts copper is a co-factor in basal APP gene regulation. The data also further supports the role of APP to function in copper efflux/detoxification. The elucidation of the copper-regulation mechanisms of APP in human fibroblasts may provide new targets in developing therapeutic strategies in the treatment of AD that are designed to reduce the expression of the APP gene and the ensuing production of Aβ. These findings also suggest that current phase II clinical trials of copper-zinc chelators in the treatment of AD (12,44,45) may have an important secondary effect of down-regulating APP gene expression.

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FIG. 1. Expression of the MNK protein and intracellular metal levels in human
MNK deletion and MNK transfected fibroblasts. Panel A, Western immunoblot
detection of MNK. 50µg of whole cell lysates were examined for MNK protein
eexpression by immunoblotting with a specific polyclonal antibody to the N-terminal
region of MNK (24). Representative immunoblots from four identical experiments
are shown. Panel B, Radioactive copper isotope, $^{64}$Cu, was used to assess the copper
levels of fibroblast cell lines. MNK deletion, vector only control, MNK transfected A,
MNK transfected B and normal human fibroblasts maintained $0.88 \pm 0.06$, $0.95 \pm
0.02$, $0.07 \pm 0.01$, $0.08 \pm 0.01$ and $0.44 \pm 0.03$ pmol Cu/mg protein, respectively.
Bars represent the mean ± SEM of incubation time points 16, 24 and 36 hours pooled
from 4 separate triplicate experiments (ANOVA, Bonferroni’s post test; **$P < 0.001$,
* $P < 0.01$ compared to normal human fibroblasts; ***$P < 0.001$ compared to MNK
deletion). Panel C, Atomic absorption analysis was used to determine the copper
content of fibroblast cell pellets. MNK deletion, vector only control, MNK transfected
A, MNK transfected B and normal human fibroblasts contained $34.7 \pm 1.3$, $31.7 \pm 3.1$,
$4.3 \pm 0.14$, $4.2 \pm 0.11$ and $19.4 \pm 1.8$ pg Cu/10$^6$ cells, respectively. Bars represent the
mean ± SEM of four separate cell pellets measured in triplicate (ANOVA,
Bonferroni’s post test; **$P < 0.001$, * $P < 0.01$ compared to normal human
fibroblasts; ***$P < 0.001$ compared to MNK deletion). Panel D, Atomic absorption
analysis was used to determine the iron content of fibroblast cell pellets. Bars
represent the mean ± SEM of four separate cell pellets measured in triplicate
(ANOVA, $P > 0.05$). Panel E, Atomic absorption analysis was used to determine the
zinc content of fibroblast cell pellets. Bars represent the mean ± SEM of four
separate cell pellets measured in triplicate (ANOVA, $P > 0.05$).
Running Title: Copper depletion down-regulates APP gene expression

Fig. 2. Endogenous basal APP protein and APP mRNA expression in MNK deletion and MNK transfected fibroblasts. Panel A, Expression of endogenous APP protein in various cell lines. Immunostaining was performed with an antibody to the Aβ region of APP (32). Cultured human neuron cells (upper left panel) were used as a positive control to detect endogenous APP levels. Images were taken under identical exposure conditions by confocal microscopy. Panel B, Western immunoblot of endogenous APP. We examined 50µg of whole cell lysates by immunoblotting with an antibody to the Aβ region of APP. APP isoforms were detected. Purified human APP695 isoform ectodomain was loaded as a control (35). Representative immunoblots from four identical experiments are shown. Panel C, Northern blot analysis of APP expression. 5µg of total mRNA isolated from cell lines was probed with human APP cDNA probe (APP, upper panel). Reprobing with glyceraldehyde phosphate dehydrogenase “housekeeping” cDNA probe (GAPDH, lower panel) served as a control for loading and was used for normalisation. Representative northern blots from four identical experiments are shown. Panel D, Normalisation of APP expression against parental MNK deletion fibroblasts as the control cell line. Bars are means ± SEM for four independent experiments (ANOVA, Bonferroni’s post test, ***P < 0.001 compared to MNK deletion).
Running Title: Copper depletion down-regulates APP gene expression

FIG. 3. Promoter Analysis of the APP gene in human MNK deletion and MNK transfected fibroblasts. Panel A, Schematic Representation of 5′ APP-CAT Promoter Constructs. □ pBLCAT3, basic promoter-less CAT construct. □ pβBIV, −3416 to +104 5′ APP-CAT promoter fusion construct. □ pβPB, −1131 to +104 APP-CAT promoter fusion construct. □ pβHB, −490 to +104 APP-CAT promoter fusion vector. Putative copper and metal-response elements are shown (● CuRE; ■ Ace/AmtRE; ▲ MRE; △△△△ Ace/AmtRE-like and MRE-like rich regions). Panel B, Promoter analysis of MNK deletion and MNK transfected fibroblasts in basal media. MNK transfected A and MNK transfected B cell lines demonstrated a significant decrease in APP promoter activity with pβBIV, pβPB and pβHB constructs when compared to MNK deletion (ANOVA, Bonferonni’s post test; *P < 0.001 for pβBIV, #P < 0.001 for pβPB, ^P < 0.001 for pβHB. Bars represent the mean ± SEM of four duplicate experiments). Panel C, Promoter analysis of MNK transfected cells in copper-supplemented media. No significant difference in APP promoter activity was observed between basal (0.78 µM Cu) and copper-supplemented (50, 100, 250 and 500 µM Cu) media for pβBIV, pβPB and pβHB constructs (ANOVA, P > 0.05, Bars represent the mean ± SEM of four duplicate experiments). Panel D, Promoter analysis of MNK deletion cells in copper-chelated media. A significant decrease in APP promoter activity was observed between basal and copper-chelated (5, 10, 50 µM diamsar) media for the pβBIV, pβPB and pβHB constructs (ANOVA Bonferonni’s post test; *P < 0.001 for pβBIV, #P < 0.001 for pβPB, ^P < 0.001 for pβHB. Bars represent the mean ± SEM of four duplicate experiments).
**Running Title:** Copper depletion down-regulates *APP* gene expression

**TABLE I**

*Description of fibroblast cell lines and their MNK expression*

*MNK transfected* cell lines are two independently derived clones isolated from the stable transfection of MNK into the *MNK deletion* cell line (25).

* Refer to Experimental Procedures for further information.

| Name         | Cell Line | Description*                                      | MNK          |
|--------------|-----------|---------------------------------------------------|--------------|
| Immortalised human | Me32aT22/2L | fibroblast isolated from classical Menkes disease patient | None detected |
| *MNK deletion* | pCMB77     | Me32aT22/2L stably transfected with empty vector | None detected |
| *Vector only* | MNK A12-H9 | Me32aT22/2L stably transfected with MNK expression vector | Over-expressed |
| *MNK transfected A* | MNK C3-C1 | Me32aT22/2L stably transfected with MNK expression vector | Over-expressed |
| *Normal human* | GM2069     | Immortalised normal human fibroblast              | Wild-type    |
Running Title: Copper depletion down-regulates APP gene expression

TABLE II

List of potential Ace/AmtRE, CuRE, MRE and like sequences in the human and rhesus monkey APP promoters.

The human APP gene promoter (GenBank Accession Number D87875) and rhesus monkey APP promoter sequences (GenBank Accession Number AF067971) were taken between the upstream and downstream BamHI sites relative to the transcription start site and searched using the Transcription Element Search System (31). Promoters were searched for copper response elements (28) (CuRE consensus sequence 5' WWWTTTGCKCR 3'); ACE1/AMT1 metal response elements (29) (Ace/AmtRE consensus sequence 5' THNNGCTG 3'); and metal response elements (30) (MRE consensus sequence 5’ TGCRCNC 3’). In addition promoters were searched for ACE1/AMT1-like sequences (ALS), with no more than one base mismatch to the first two ACE1/AMT1 consensus residues (5’ THNNGCTG 3’); and MRE-like sequences (MLS), with no more than one base mismatch from the last three MRE consensus residues (5’ TGCRCNC 3’). (The negative numbers indicate upstream sequence relative to the transcription start site. Reverse-orientation sites are in italics. Forward-orientation sites are in boldface).

| Potential Site | Human          | Rhesus Monkey |
|----------------|----------------|---------------|
| CuRE          | -1630          | -1678         |
| Ace/AmtRE     | -3417,-2622,-1175,-533,12 | -3322,-3228,-3160,-2555,-584,14 |
| MRE           | -2410,-1998,-182,-160,-113 | -3172,-1331,-165 |
| ALS           | -3704,-3579,-3385,-3255,-3083,-2845, -2625,-2418,-2160,-2006,-1839,-1785, -1531,-1520,-1218,-1214,-1150,-1137, -1075,-885,-400,-51,-49 | -3128,-3006,-2590,-2370,-2367,-2176, -2055,-1891,-1888,-1834,-1579,-1552, -1253,-1190,-1177,-450,-267,-103, -101 |
| MLS           | -3616,-3338,-2927,-2924,-2655,-2143, -2140,-1971,-1712,-1656,-1619,-1579, -1175,-1147,-1142,-1139,-619,-272, -179,-157,42 | -3359,-3081,-2672,-2669,-2400,-2159, -2156,-2047,-2020,-1779,-1704,-1667, -1194,-1187,-1182,-1179,-322,-231,-228, -209 |
**Running Title:** Copper depletion down-regulates *APP* gene expression

**TABLE III**

*Copper levels in MNK transfected cell lines after copper-supplementation*

*MNK deletion, vector only* control and *MNK transfected* cells were incubated for 48 hours in basal or copper-supplemented media containing trace amounts of $^{64}$Cu. *Values for total copper represent the mean ± SEM amount of copper accumulated in cells after 48 hours in basal or copper-supplemented 10% BME media from four separate triplicate experiments (pmol Cu/mg protein).*

| Copper-supplement (µM Cu) | MNK deletion | Vector only | MNK transfected A | MNK transfected B |
|---------------------------|--------------|-------------|-------------------|-------------------|
| Basal (0.78)              | 3.70 ± 0.43  | 4.36 ± 0.31 | 0.44 ± 0.13       | 0.54 ± 0.17       |
| 50                        | —            | —           | 2.03 ± 0.72       | 2.12 ± 0.93       |
| 100                       | —            | —           | 4.82 ± 1.69       | 5.20 ± 1.76       |
| 250                       | —            | —           | 14.8 ± 6.6        | 15.5 ± 5.6        |
| 500                       | —            | —           | 81.6 ± 4.1        | 90.7 ± 7.8        |
Figure 1
Figure 2

(A) Images showing different cell types: Normal Human, MNK Transfected A, MNK Transfected B, MNK Deletion, Vector Only.

(B) Western blot analysis of APP(s) with bands at 185 kDa, 98 kDa, 52 kDa, and 31 kDa.

(C) Western blot showing APP and GAPDH bands.

(D) Bar chart showing normalized APP mRNA expression with significant differences indicated by asterisks.
Figure 3
Copper depletion down-regulates expression of Alzheimer's disease Amyloid-β precursor protein gene
Shayne A. Bellingham, Debomoy K. Lahiri, Bryan Maloney, Sharon La Fontaine, Gerd Multhaup and James Camakaris

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