CHARACTERIZATION OF THE EXPRESSION AND REGULATION OF THE MENKES PROTEIN IN AN ADRENOCORTICOTROPIC TUMOR CELL LINE AND RAT ENDOCRINE TISSUES

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ABSTRACT

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To ensure copper homeostasis, organisms express copper chaperones and the Menkes copper transporter (MNK). Mutations within the MNK gene result in MNK disease, characterized by neurodegeneration, cutis laxa, and hypopigmentation. These symptoms are attributed to a deficiency in copper delivery to cuproenzymes, such as peptidylglycine α-amidating monooxygenase (PAM). Fibroblasts transfected with MNK have copper-dependent MNK localization in the trans-Golgi network (TGN). Since MNK localization in neuroendocrine cells has not been investigated, this study examined MNK localization in adrenocorticotropic tumor wild type (AtT-20 WT) and PAM (AtT-20 PAM-1) cell lines by immunofluorescence microscopy, subcellular fractionation, and Western blot analyses. To analyze the effect of copper levels on MNK localization, cells were treated with CuCl₂, the copper chelator, BCS, and BCS/CuCl₂. Immunofluorescence microscopy confirmed that MNK localization is in the TGN in WT and PAM-1 control and BCS treated cells, while in CuCl₂ treated cells, MNK showed TGN and diffuse staining throughout the cytoplasm. Sucrose density gradients from control cells showed MNK localization in TGN and secretory granules. A wider distribution of MNK into lighter fractions in these gradients was observed following copper treatments, indicating MNK trafficking is copper-dependent. In addition, developmental differences in MNK expression in rat brain and endocrine tissues also were examined by Western blot analyses and RT-PCR. In adrenals, MNK protein expression increased, whereas MNK mRNA remained unchanged. In the pituitary, hippocampus and cerebellum, MNK protein
and mRNA decreased, while in the atria, they remained unchanged. Therefore, MNK mRNA was differentially expressed during development. Thus, little parallel between expression of MNK protein and mRNA was observed from postnatal day 3 to adult. Since secretagogues alter PAM expression and PAM activity requires copper delivery from MNK, this study also examined the MNK expression regulation by secretagogues. While MNK secretion was not stimulated, it was regulated by secretagogues. MNK expression was increased after treatment with PMA, CRH and cAMP, with a slight decrease after BaCl₂ treatment. Overall, these studies have begun to shed light on how the endogenous MNK protein functions in cells expressing a cuproenzyme dependent on copper delivery from MNK.
I dedicate this manuscript to Drs. Josephine and Fredrick Collaco, for their continuous love, support, encouragement and faith in me.
I wish to extend my thanks to everyone who lent their support towards the completion of this dissertation. I would like to take this opportunity to express my appreciation to my advisor, Dr. Tami Steveson for her time spent in designing experiments, discussing results and trying to figure out why we care about these experiments in the context of the bigger picture. Thank you for all your patience, guidance, support, humor, excitement and encouragement throughout my studies.

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LIST OF ABBREVIATIONS

ACTH – Adrenocorticotropic hormone
ATP7A– Menkes protein in humans
Atp7a– Menkes protein in mice
ATP7B – Wilson’s protein
AtT-20 – Adrenocorticotrope tumor cell line
AtT-20 PAM-1 – Adrenocorticotrope tumor cell line transfected with PAM-1
B1 – 1st basal period
B2 – 2nd basal period
BaCl2 – Barium chloride
BCA – Bicinchoninic acid
BCS – Bathocuproinedisulfonic acid
BSA – Bovine serum albumin
cAMP – Cyclic-adenine monophosphate
CGA – Chromogranin A
CHO – Chinese hamster ovary
CO2 – Carbon Dioxide
Cox17 – Cytochrome c oxidase
CRH – Corticotrophic releasing hormone
CuCl2 – Copper Chloride
Cy3-DAM – Cy3-conjugated donkey antibody to mouse IgG
DABCO – 1,4-diazabicyclo(2.2.2.) octane
DBM – Dopamine β-monooxygenase
DEPC – Diethylpyrocarbonate
DMEM – Dulbecco’s Modified Eagle’s Medium
ECL – Enhanced Chemiluminescence
EDTA – Ethylenediaminetetra acetic acid
FCS – Fetal Calf Serum
FSH – Follicle stimulating hormone
GAR-FITC – Fluorescein isothiocyanate-conjugated goat antibody to rabbit IgG
GH – Growth hormone
GHRH – Growth hormone releasing hormone
GnRH – Gonadotrophin releasing hormone
H – Homogenate
HAH1 – Human atox-1 homologue
hCtr1 – Human copper transporter 1
IgG – Immunoglobulin G
LH – Leuteinizing hormone
MNK – Menkes protein
Mo^{br} – Mottled brindled mice
mRNA – Messenger Ribonucleic acid
OD – Optical density
P – Pellet
p65 – Synaptotagmin
PAGE gel – Polyacrylamide gel electrophoresis gel
PAL – Peptidylglycine α-hydroxylglycine α-amidating lyase
PAM – Peptidylglycine α-amidating monooxygenase
PBS – Phosphate buffered saline
PC1 – Prohomone convertase 1
PCR – Polymerase chain reaction
PHM – Peptidylglycine α-hydroxylating monooxygenase
PMA – Phorbol myristate acetate
PMSF – Phenylmethylsulfonyl fluoride
POMC – Proopiomelanocortin
PRL – Prolactin releasing hormone
RT – Room temperature
RT-PCR – Real Time Polymerase chain reaction
S – Supernatant
S – Stimulation
SDS – Sodium dodecyl sulphate
SNAP-25 – Soluble N-ethyl-maleimide-sensitive factor attachment protein
SOD – Superoxide dismutase
TAE – Tris acetate ethylenediaminetetra acetic acid
TGN – Trans Golgi network
TMT – Tes Mannitol Triton
TRH – Thyrotropin releasing hormone
TSH – Thyroid stimulating hormone
TTBS – Tris buffered saline containing Tween 20
WT – Wild type
INTRODUCTION

Cellular function in all living organisms is dependent on the essential trace element, copper. The link between copper and hemoglobin metabolism provides an illustration of the essential nature of copper. As early as 1928, it was demonstrated that the effects of anemia could be reversed in rats if they were fed ash, which contained copper sulfide (Hart et al., 2002). The link between copper and specific cuproenzymes that require copper for growth and development further illustrate this point. It has been shown that reduced levels of copper can alter the function and activity of cuproenzymes. For example, the delivery of copper to the cuproenzyme, tyrosinase, can lead to a decrease in the synthesis of melanin (Sanchez-Ferrer et al., 1995). Copper can undergo redox reactions, and these copper ions can serve as cofactors for proteins. Although copper is essential for proper cellular function, it also can be damaging if its homeostasis is not maintained. For example, in the presence of oxygen, copper can participate in reactions that produce hydroxyl radicals, which can cause cellular damage, including the peroxidation of membranes, the oxidation of proteins, and the cleavage of DNA and RNA (Halliwell and Gutteridge, 1984; Camakaris et al., 1999; Tapiero et al., 2003). Excess copper can influence gene regulation, for example for the human estrogen receptors, Cu$^{2+}$ can replace Zn$^{2+}$ altering the DNA binding domains and leading to the disruption of endocrine functions and hormone signaling (Predki and Sarkar, 1992). In general, copper is crucial for biological reactions; however, copper balance must be maintained appropriately in the body.

In humans, dietary needs are met through a variety of foods, including legumes, potatoes, grains, and shellfish (Linder and Hazegh-Azam, 1996; Turnland et al., 1998). Normal dietary intake of copper ranges from 0.6 to 1.6 mg per day; uptake of over 10 mg can lead to copper toxicity (Trumbro et al., 2001). Figure 1 illustrates the absorption of copper through the gut and
Dietary copper is absorbed by the enterocytes of the small intestines. Copper can then bind to copper-histidine or albumin in the portal blood vein. Human copper transporter 1 (hCTR1) transports copper into mucosal cells. Copper can also bind to ceruloplasmin in the liver and be secreted back into the blood, where it can be transported to other tissues and organs. (Reproduced from Pêna et al., 1999)
transport of copper within the human body. The cells in the small intestine of the human digestive tract, specifically the enterocytes, absorb 55-75% of all ingested copper (Tapiero et al., 2003). Copper is transported across the brush-border of these mucosal cells, where it is then exported across the basolateral membrane into the bloodstream. Metallothionein proteins, which bind metals and aid in the transport of ions, also can bind excess copper that is not transported on to its next destination. From the intestinal tract to the liver, copper binds to serum albumin and histidine in the blood for transport. Human copper transporter 1 (hCTR1) has been shown to aid in the transport of copper into the liver. The liver plays a key role in homeostasis, storage, and distribution of copper. Hepatocytes in the liver process, store, and secrete copper. Copper then is incorporated into the copper binding protein, ceruloplasmin, for distribution to peripheral target tissues, while excess copper is secreted into the bile (Harrison and Dameron, 1999; Pêna et al., 1999; Mercer, 2001; Mercer and Llanos, 2003). Once cells uptake copper, the chaperones within cells deliver copper to the appropriate organelles.

Previous studies in yeast and bacteria have yielded information on how copper is absorbed, distributed and used by the cell as well as the cellular copper chaperones involved in the distribution of copper (Solioz et al., 1994; Hassett and Kosman, 1995; Harris et al., 1998; Huffman and O’Halloran, 2001). For example, in gram-positive bacteria, within the cop operon of Enterococcus hirae, five genes (cop X, Y, Z, A and B) have been shown to play a role in copper homeostasis (Odermatt et al., 1992; Magnani and Solioz, 2005). Furthermore, as proteins, the cop A and B chaperones transport copper into the cell where they are involved in the trafficking of copper under high and low levels of copper. In addition, cop A and cop B share the conserved amino acid sequence, DKTG, which is characteristic of P-type ATPases. More recently, studies have progressed to understanding copper homeostasis in eukaryotic systems.
At the eukaryotic level, copper transport and distribution has been extensively studied in yeast (Pêna et al., 1998). In *Saccharomyces cerevisiae*, the copper transporter, copper transporter 1, has been shown to transport copper into the cell (Puig et al., 2002; Petris et al., 2003). Moreover, the yeast metallochaperone, anti-oxidant 1 (Atx1), has been observed to interact with Ccc2, a copper transporter, to deliver copper to vesicles in the secretory pathway (Lin et al., 1997; Huffman and O’Halloran, 2000).

Evolutionary and biochemical studies have demonstrated that yeast and human copper binding chaperone proteins share a high degree of homology (Koch et al., 1997; Harris 2000; Field et al., 2002). Interestingly, hCTR1 shares 29% homology with yeast Ctrl1 and also has been shown to be involved in copper uptake (Zhou and Gitschier, 1997; Klomp et al 2002; Puig et al., 2002). In addition, yeast Atx1 is similar to human Atox1 (Klomp et al., 1997). When a human cDNA library was probed with Atx1, the human homolog, Atox1 or HAH1 (for human ATX homolog 1), was identified and the isolated protein of HAH1 was found to be 47% identical to the Atx1 protein (Klomp et al., 1997). Both of these chaperones contain MTCXGC copper binding domains and interact with P-type ATPases. In yeast, Atx1 interacts with Cce2p, a membrane bound protein, to transport copper into the Golgi, while HAH1 in mammals interacts with ATP7B, the Wilson’s disease protein (Lin et al., 1997). In both *S. cerevisiae* and humans, the cytochrome c oxidase chaperone (COX 17) is used for transporting copper to cytochrome c oxidase in mitochondria (Amaravadi et al., 1997; Beers et al., 1997). In addition, LYS7 in yeast and the copper chaperone (CCS) in humans are both involved in delivering copper to the enzyme, superoxide dismutase (SOD) (Culotta et al., 1997). The identification of these copper chaperones in yeast and humans has helped researchers begin to elucidate the complex mechanisms involved in copper homeostasis and trafficking.
Since copper is required for several enzymatic reactions and in its free radical state (Cu I or Cu\(^+\)) can be damaging to cells, copper metabolism and homeostasis in humans is vitally important (Figure 2) (Koch et al., 1997; Peña et al., 1999). Specific chaperones and enzymes have been identified to transport copper in the cell (Table 1). At the cellular level, copper is reduced from Cu II to Cu I, where Cu I binds to the copper transporter, hCtr1, for transport across the plasma membrane and delivery into the cell (Lee et al., 2002). Since an accumulation of Cu I ions can lead to the production of reactive oxygen species, which can lead to cellular damage, copper then is distributed to cytosolic chaperones including the CCS1, Cox17, and HAHD1 (Fig. 2). Cox17 delivers copper to cytochrome c oxidase in the mitochondria. Cytochrome c oxidase is involved in electron transport, where it reduces oxygen to water as well as producing a membrane potential. CCS delivers copper to the copper/zinc superoxide dismutase (SOD) in the cytosol, where SOD is involved in free radical detoxification, for example, in the conversion of superoxide anions to peroxide. In addition, Atox1 delivers copper to the Menkes (MNK or ATP7A) and Wilson (ATP7B) proteins. One of the roles of the MNK protein is to deliver copper to cuproenzymes, such as peptidylglycine \(\alpha\)-amidating monooxygenase (PAM), as well as to efflux excess copper from the cell. An inability to deliver copper to enzymes requiring copper could produce devastating results in an organism as shown in Table 1 (Peña et al., 1999; Mercer, 2001; Fatemi and Sarkar, 2002; Mercer and Llanos, 2003; Steveson et al., 2003; Rossi et al., 2004).

The Menkes (ATP7A) and Wilson’s (ATP7B) proteins are members of the cation translocating P-type adenosine triphosphatase (ATPase) family (Lutsenko and Kaplan, 1997; Lutsenko and Petris, 2003). Other P-type ATPases include sodium/potassium and calcium pumps (Lutsenko and Kaplan, 1994; Moller et al., 2005). P-type ATPases can drive a variety of ions
Figure 2: Copper Transport in a Cell. Step 1. Copper is reduced from Cu$^{2+}$ to Cu$^+$.

Step 2. The copper ion is taken up by human copper transporter 1 (hCtr1), a plasma membrane protein with high affinity for Cu$^+$. Step 3. Cellular copper binds to the chaperone, Cox17, for delivery to cytochrome c oxidase (Cyt Ox) in the mitochondria. Step 4. Cellular copper binds to the copper chaperone, CCS1, for delivery to the cytosolic enzyme, superoxide dismutase (SOD). Step 5. HAH1 delivers copper to the Menkes protein, ATP7A, which is localized to the trans-Golgi network (TGN). Step 6. ATP7A helps direct copper to the secretory pathway for the delivery of copper to cuproenzymes, such as peptidylglycine α-amidating monooxygenase (PAM). (Reproduced from Steveson et al., 2003)
<table>
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Table 1: Copper Requiring Enzymes, Their Localization in the Cell. (Modified from Pêna et al., 1999; Mercer, 2001).
across cellular membranes including $\text{Ca}^{2+}$, $\text{K}^+$, $\text{Na}^+$ and $\text{Cu}^+$ (Palmgren and Axeslen, 1998). All P-type ATPases identified to date are composed of several transmembrane domains (TMD), which localize the protein in the membrane and are separated by cytoplasmic domains and extracellular loops. In general, P-type ATPases contain four main motifs: a phosphorylation domain (DKTG), a phosphatase domain (TGEA/S), an ATP binding domain (TGDN) and a metal binding domain (CXXC) (Lutsenko et al., 1995; Moller et al., 1996). Furthermore, P-type ATPases use the phosphorylation and dephosphorylation reactions of ATP to undergo conformational changes, create channels and move ions across membranes (Lutsenko et al., 1995).

As a member of the P-type ATPase transporter family, MNK protein binds copper, a heavy metal (Mercer, 1998). Figure 3 illustrates the predicted topology of the MNK protein. With both its amino and carboxyl termini located in the cytosol of the cell (Figure 3), the MNK protein spans the membrane eight times with three cytosolic and four lumenal loops. At the amino terminus of the MNK protein, there are six heavy-metal binding domains (GMXCXXC, where X is any amino acid) to which one ion can bind. Studies have examined the role of the six metal binding sites and the role of MNK protein trafficking in response to copper (Goodyer et al., 1999; Strausak et al., 1999; Voskoboinik et al., 1999). It is still not clear if all six metal binding sites must be occupied with copper to serve as a mechanism for sensing copper levels and thus effluxing excess copper from the cells. Mutations and deletions of the MNK protein metal binding domains have demonstrated that the sixth metal binding domain closest to the putative channel is sufficient to promote the trafficking of the MNK protein from the trans-Golgi network (TGN) to the plasma membrane. Furthermore, the sequence alignment of the first and fourth copper binding sites from the copper ATPases in bacteria, human and mouse respectively
The Predicted Topology of The Menkes Protein

Figure 3: The Predicted Topology of the Menkes Protein (MNK). Shown are the heavy-metal binding domains (1, CxxC), the putative glycosylation site (2, N), the phosphatase domain (3, TGES), the putative copper transduction site (4, CPC), the phosphorylation domain (5, DKTG), the ATP binding domain (6, TGDN), the Mg²⁺ binding “hinge” region, (7, GDGNVD), and the internalization motif (8, LL). (Modified from Horn and Tümer, 1999)
have shown that the CXXC sequence is conserved across species, suggesting that this site is involved in heavy metal transport (Vulpe et al., 1993; Odermatt, et al., 1994).

As is typical of many P-type ATPases, the 1500 amino acid residue MNK protein contains the following motifs: an ATP-binding domain (TGDN), a phosphatase domain (TGEA), a phosphorylation domain (DKTG), a hinge region/Mg$^{2+}$ binding domain (GDGNVD), an internalization domain (LL), a putative copper transduction site (CPC), and a potential glycosylation site (N) (Fig. 3) (Harris et al., 1998; Horn and Tümer, 1999; Mercer, 2001; Voskoboinik et al., 2001; Fatemi and Sarkar, 2002; Lutsenko and Petris, 2002). Like other P-type ATPases, it has been proposed that MNK binds to ATP in the ATP binding domain and hydrolyzes ATP to generate a γ-phosphate, which forms an acyl phosphorylated intermediate from the aspartic acid that is undergoing phosphorylation, resulting in the formation of a putative channel. The CPC domain allows the movement of ions across a membrane. Reversible dephosphorylation by the phosphatase domain is predicted to return the MNK protein to its original conformation. Voskoboinik and colleagues (2001) have demonstrated that this cycle begins with the binding of copper, followed by the binding of ATP and the phosphorylation cycle.

Since one of the roles of the MNK protein is to efflux excess copper from the cell, studies have examined the importance of the formation of the phosphorylated catalytic intermediate in the trafficking of the MNK protein (Petris et al., 2002). Through mutagenesis experiments, involving both the phosphorylation and phosphatase domains, Petris and colleagues (2002) demonstrated in Chinese hamster ovary (CHO) cells that MNK protein localization was altered if the acyl-phosphorylated intermediate was not formed. Furthermore, in yeast, it has been shown using wild-type (WT) and mutant MNK protein that the aspartic acid residue in the DKTG
domain must be phosphorylated for ATPase catalytic activity; this motif is also necessary for copper transport (Voskoboinik et al., 2001).

Studies also have been aimed at examining how the various domains are involved in the maintenance of the subcellular localization of the MNK protein. A carboxyl-terminal dileucine motif (L1487 and L1488) has been determined to be the internalization signal that allows MNK to enter the endosomal pathway from the cell surface (Petris et al., 1998; Petris and Mercer, 1999; Lane et al., 2004). A TGN retention motif also has been identified in a MNK recombinant protein in Exon 10, which encodes the transmembrane domains 3 and 4 (Francis et al., 1998). In general, these domains are involved in helping to maintain copper homeostasis.

Several studies have demonstrated that the MNK protein is localized to the TGN in the secretory pathway, where it has been demonstrated to deliver copper to enzymes such as peptidylglycine α-amidating monooxygenase (PAM) and lysyl oxidase (Petris et al., 1996; Yamaguchi et al., 1996; Dierik et al., 1997; Steveson et al., 2003). More specifically, studies have examined the subcellular localization of the MNK protein in a variety of cell types, including CHO cells, mottled brindled (Mo<sup>br</sup>) mice cells and human fibroblasts transfected with MNK (Dierick et al., 1997; Petris et al., 1998; La Fontaine et al., 1999; Petris and Mercer, 1999; Mercer, 2001; Lutsenko and Petris, 2002). For example, using subcellular fractionation and immunofluorescence microscopy, it was determined that MNK was localized to the TGN at steady-state in CHO cells (Petris and Mercer, 1999). Furthermore, a study of Rab GTPase proteins, which are localized to endosomal compartments, and the MNK protein in transfected CHO cells has demonstrated that in response to copper, the MNK protein traffics from the TGN to the plasma membrane in endosomes (Pascale et al., 2003).
Since another role of the MNK protein is to efflux excess copper from the cell and since MNK is a copper transporter protein, it was hypothesized that the localization of the MNK protein in a cell could be different based upon copper levels in the medium. In fibroblasts transfected with MNK and cultured in medium containing high levels of copper (200 µM), the MNK protein was observed to traffic from the TGN to endosomes and the plasma membrane. However, when copper concentrations were reduced to basal levels (2-3 µM), the MNK protein was observed to move back from the plasma membrane to the TGN. Therefore, the localization and trafficking of the MNK protein was determined to be copper dependent (Figure 4) (La Fontaine et al., 1998a; La Fontaine et al., 1998b; Voskoboinik et al., 1998; Suzuki and Gitlin, 1999; Pascale et al., 2003; Pase et al., 2004).

MNK mRNA and protein is expressed in most tissues, including the heart, lungs, brain, skeletal muscle, kidney, and pancreas with low expression levels detected in the liver (Vulpe et al., 1993; Paynter et al., 1994). For example, in WT mice, the MNK protein and mRNA were detected with the greatest expression in the pituitary and adrenal glands, and with lesser expression in the brain cortex, hypothalamus, and atrium (Steveson et al., 2003). Furthermore, Niciu and colleagues (2006) have begun to study the expression of the MNK gene (ATP7A or Atp7a) in postnatal brain regions. This study demonstrated that the MNK protein is developmentally regulated as well as expressed differentially in various regions in the brain. A recent study also has examined the expression of the MNK protein in the olfactory system throughout neuronal development and has shown that the MNK protein is developmentally expressed in axon extensions and during synaptogenesis (El Meskini et al., 2005). Lastly, a connection between copper homeostasis and the central nervous system has been observed showing that the activation of NMDA receptors and the release of copper cause the movement of
TRAFFICKING OF MNK

Figure 4: Diagram of the Trafficking of the MNK Protein in Response to Copper Levels. In cells treated with high levels of copper, the MNK protein moves from the TGN to the plasma membrane. In cells treated with a copper chelator, MNK moves from the plasma membrane back towards the TGN. (Modified from Mercer, 2001).
the Menkes protein in hippocampal neurons (Schlief and Gitlin, 2006). Since MNK is tissue-specific and developmentally regulated, further analyses in patients with mutated MNK could lead to a better understanding of protein function.

Mutations in copper transporter proteins, namely MNK (ATP7A) or ATP7B, can lead to either Menkes disease or Wilson’s disease, respectively. These two proteins share a 55% amino acid sequence identity (Fatemi and Sarkar, 2002). Wilson’s disease is an autosomal recessive disorder that occurs in one of every 30,000 births and results in an accumulation of high levels of copper in the liver due to a lack of the biliary excretion of copper (Figure 5) (Ferenci et al., 2003; Cater et al., 2006). Menkes disease is an X-linked recessive neurodegenerative disorder that occurs in one of every 250,000 to 300,000 births (Tonnesen et al., 1991; Kaler, 1994). Three types of Menkes disease (mild Menkes disease, classical Menkes disease, and occipital horn syndrome) have been well characterized (Byers et al., 1980; Procopis et al., 1981; Danks, 1988; Seidel et al., 2001). Menkes disease is characterized by copper not being transported out of the liver into the blood, which leads to decreased copper levels in the brain, tissues and organs (see Fig. 2). These reduced copper levels are believed to cause the hair abnormalities, connective tissue disorders, neuronal damage and early childhood death observed in patients diagnosed with Menkes disease (Kaler, 1998; Mercer, 1998; Kim et al., 2003). Between the age of two and three months, hair abnormalities, such as de-pigmentation, coarse, fine and twisted hair, referred to as “kinky” or “steely” (pili totti) hair, have been observed in Menkes patients (Danks et al., 1972). The faces of Menkes patients are characterized by sagging, fat cheeks, larger than normal ears, and pale skin. The coordination of movements and breathing also are difficult for some of these patients. Skin, which is often de-pigmented, is lax and loose near the neck and trunk of the body. Often in Menkes disease patients, growth declines shortly after birth and osteoporosis can be
Dietary copper is absorbed into the small intestine and is transported into the portal vein. Copper is taken up by the liver and distributed to the blood for transport to the brain or it is bound to ceruloplasmin for transport to other tissues and organelles. In Wilson’s disease, copper accumulates in the liver and is not transported to the bile for excretion or to ceruloplasmin for distribution. In Menkes disease, copper fails to leave the small intestine and accumulates in the enterocytes. This leads to an overall reduction of copper in the brain and tissue as well as less copper being bound to ceruloplasmin. (Modified from Mercer, 2001.)
detected. Neurological defects can lead to problems with the visualization and coordination of eye movements as well as the loss of functional cerebellar matter (Gasch et al., 2002). In addition, Menkes patients have lower than normal serum copper and ceruloplasmin levels in their blood and brains as well as increased copper levels in their intestines; this also is observed in fibroblasts cultured from the Menkes disease patients (Koch et al., 1997; Prohaska et al., 1997; Mercer, 2001). Using radiography analyses to detect bone abnormalities and fractures, laboratory tests to detect low serum copper and ceruloplasmin levels, as well as physical appearance, a diagnosis of Menkes disease can be made (Herman et al, 1992). Unfortunately, death occurs early in life by age 3 to 5 in males (Bankier, 1995). Of importance is that injections of copper-histidine or copper-acetate have been shown to increase serum copper and ceruloplasmin levels (Kaler, et al., 1995; Kodama et al., 2005); however, this therapy cannot completely improve all of the damage, and death often still results.

Symptoms presented by Menkes disease patients have been hypothesized to be caused by an inability of cuproenzymes to receive copper from MNK (Mercer, 2001) (see Table 1). For example, it has been shown that MNK is involved in the delivery of copper to tyrosinase, an enzyme involved in the formation of melanin (Petris et al., 2000). In patients with MNK disease, hypopigmentation is observed, connecting the lack of copper being delivered to tyrosinase to the lowered activity of this enzyme. The cuproenzyme, lysyl oxidase, also requires copper for its role in the formation of connective tissue. Lysyl oxidase activity is decreased in Menkes disease patients, perhaps resulting in the loose connective tissue formation that is observed in these patients (Byers et al., 1980; Royce et al., 1980; Kuivaniemi et al., 1982).

Symptoms displayed by human Menkes disease patients also have been observed in mice. Mutations in the MNK gene in mice or the Atp7a locus result in the loss of pigmentation in coat
color in heterozygous female mice. These mice are known as mottled (Mo) mice. There are over 25 copper deficient Mo mice strains that have been used to study the role of Atp7a (The Jackson Laboratory, Mouse Genomic Database). The Mo^br mouse strain is the closest animal model to humans with classical Menkes disease (Cecchi et al., 1996; Kodama and Murata, 1999). Both the mutated and WT Atp7a genes have been mapped to homologous areas on the X-chromosome (Xq13) (Tonnesen et al., 1992). The Atp7a gene in Mo^br mice shares 87% homology with the human MNK gene (ATP7A) (Levinson et al., 1994; Mercer et al., 1994). The mutant Atp7a gene encodes a predicted 1491 amino acid residue protein as opposed to the 1500 amino acid residue protein for encoded by the human ATP7A gene. The mouse MNK protein is structurally similar to the human MNK protein and contains all of the same motifs (Reed and Boyd, 1997; Kodama and Murata, 1999). However, in the mouse Mo^br MNK protein, there is a six base-pair deletion, resulting in the absence of two residues (Ala^799 and Leu^800) between the fourth and fifth transmembrane domains from the predicted protein product (Grimes et al., 1997; Cunliffe et al., 2001). Similarly, in mottled variable brindled mice, which are comparable to patients with mild Menkes disease, there is a substitution of Thr for Lys at position 1037 in the ATP phosphorylation motif (Reed and Boyd, 1997).

The Mo^br and mottled blotchy (Mo^blo) mice strains have been well studied as models for the various forms of Menkes disease (Phillips et al., 1991). Mo^blo mice have symptoms similar to patients with occipital horn syndrome. The Mo^br male mice display symptoms similar to patients with classical Menkes disease, including a lack of connective tissue formation, the loss of the hair pigmentation, curly whiskers, neuronal degeneration, and early death (Hunt, 1974; Grimes et al., 1997). Camakaris and colleagues (1979) have shown that in Mo^br mice there was an accumulation of copper in the kidney and gut; however, the brain, liver and other organs
displayed a decrease in copper concentrations. In addition, as in human Menkes disease patients, the loss of activity of cuproenzymes in the $Mo^{br}$ mice might also result in the symptoms observed in Menkes disease. The $Mo^{br}$ mice also have connective tissue disorders caused by the reduction of lysyl oxidase activity and hypopigmentation caused by the loss of tyrosinase activity (Gacheru et al., 1993; La Fontaine et al., 1999). Furthermore, a decrease in cytochrome c oxidase levels in the brains of $Mo$ mice has been observed (Rezek and Moore, 1986; Yoshimura et al., 1990). Interestingly, in postnatal day 6 mottled macular mice, injections of copper have been shown to increase cuproenzyme activity in the brain (Meguro et al., 1991).

Studies also have examined the localization and expression of the MNK protein and mRNA in mutant mice (Murata et al., 1997; Murata et al., 1998; Steveson et al., 2003; Ke et al., 2006). Steveson and colleagues (2003) demonstrated that the MNK protein expression levels in WT and $Mo^{br}$ mice in the pituitary, adrenals, atria, hypothalamus and cortex were not drastically different. Ke and colleagues (2006) demonstrated that in WT mice, $Atp7a$ also was expressed in the lung, heart, liver, kidney, small intestines, and brain. In situ hybridization and Northern blot analyses of WT and macular mice, another animal model for Menkes disease, showed that transcribed $Atp7a$ gene product levels were the same in postnatal day 0, 7, 10 and 13 and that it also was expressed during development in the brain, specifically in the hippocampus, dentate gyrus, olfactory bulb nuclei, cerebellar granular cells, and chorid plexus (Niciu et al., 2006). Furthermore, Paynter and colleagues (1994) demonstrated in $Mo^{br}$ and $Mo^{blo}$ mutant mice as well as in WT mice, that MNK mRNA levels in both the kidney and liver deceased with age. Overall, this indicates that the MNK gene, mRNA and protein are all developmentally regulated and that their expression is tissue-specific.
The intracellular localization of the MNK protein in Mo\textsuperscript{br} mice could yield further information about copper transport and MNK protein trafficking within the secretory pathway. In comparison to fibroblasts and CHO cells transfected with MNK, localization studies of the mutated MNK protein in Mo\textsuperscript{br} mice fibroblasts demonstrated that the mutated MNK protein is localized in the perinuclear region in cells cultured with basal levels of copper. However, with increased copper levels added to the incubation medium (200 \( \mu \text{M} \)), the mutated MNK in Mo\textsuperscript{br} mice fibroblasts remained localized to the TGN or perinuclear region. Furthermore, this mutated MNK protein did not move towards the plasma membrane as observed for the WT MNK protein in fibroblasts transfected with MNK that were incubated with increased copper levels in the medium (La Fontaine \textit{et al.}, 1999).

Studies also have examined the role of the WT or mutated MNK protein in the delivery of copper to enzymes within the secretory pathway (Petris \textit{et al.}, 2000; Lutsenko and Petris, 2003; Steveson \textit{et al.}, 2003). For example, it has been demonstrated that in yeast, \textit{S. cerevisiae}, the P-type ATPase, Ccc2p, is involved in transporting copper from the cytosol to the TGN for further delivery to Fet3, a yeast homolog for ceruloplasmin. It also has been demonstrated that the MNK protein can deliver copper to FetP, suggesting that the MNK protein might play a role in delivering copper to enzymes within the Golgi (Fu \textit{et al.}, 1995; Yuan \textit{et al.}, 1995; Payne and Gitlin, 1998). Furthermore, Qin and colleagues (2006) have demonstrated that the MNK protein is involved in the activation of the cuproenzyme, SOD3, in fibroblasts from male mutant mice and that MNK protein delivers copper to this enzyme through interactions in the TGN. Lastly, MNK protein has been shown to deliver copper to the secretory granule amidating cuproenzyme, PAM, in the TGN (Steveson \textit{et al.}, 2003). Figure 6 illustrates how copper is transported across the plasma membrane into the cell and how PAM might acquire copper from the MNK protein.
Figure 6: A Proposed Diagram of How Copper is Transported Across the Plasma Membrane into the Cell and How PAM Might Acquire Copper from the MNK Protein.
El Meskini and colleagues (2003) demonstrated in yeast mutants of PAM, that the MNK protein delivers copper into the secretory pathway, thus activating PAM by copper. These studies indicate that besides effluxing excess copper from cells, the MNK protein also plays a role in the delivery of copper to cuproenzymes.

The copper dependent enzymes, dopamine β-monooxygenase (DBM) and PAM, are localized to secretory granules (Eipper et al., 1993a; Oyarce and Eipper, 1993; Oyarce and Eipper, 1995; Oyarce and Eipper, 2000). DBM is involved in the synthesis of catecholamines from tyrosine in adrenal medullary cells, while. PAM is involved in the amidation of over 50% of all neuropeptides and hormones (Stewart and Klinman, 1988). This amidation process is a two-step reaction, which begins in the TGN but occurs primarily in secretory granules. Studies of both DBM and PAM have been used to determine the regulation and sorting processes of proteins in the regulated secretory pathway (Milgram et al., 1997; Steveson et al., 1999; Oyarce et al., 2001; Steveson et al., 2001). Both DBM and PAM are synthesized and packaged along with their substrates into secretory granules, which undergo regulated exocytosis in response to an external signal.

Peptides that can be α-amidated by PAM are expressed in both vertebrates and non-vertebrates. Examples include; gonadotropin and thyrotropin-releasing hormone (GnRH and TRH), substance P, neuropeptide Y, oxytocin, vasopressin, gastrin, and calcitonin (Prigge et al., 2000). This post-translational α-amidation reaction is necessary for converting inactive intermediate peptides into bioactive ones (Eipper et al., 1992a,b). For example, the α-amidation of the corticotropin-releasing hormone (CRH) causes an increase in its ability to bind to its receptors by preventing the ionization of its carboxyl-terminal; therefore, CRH becomes more
PAM is a bifunctional enzyme that modifies the carboxyl-terminal glycine of a peptide in a two-step reaction that results in the formation of an α-amidated peptide (Fig. 7) (Ouafik et al., 1992). As shown in Figure 7, PAM-1, the largest form of the protein, with an apparent molecular mass of at 120 kDa, is made up of two functional catalytic domains, peptidyl α-hydroxylating monooxygenase (PHM) and peptidyl α-hydroxyglycine α-amidating lyase (PAL), which are connected by a non-catalytic domain, Exon A, a transmembrane domain (TMD) and cytoplasmic domain (CD) (Eipper et al., 1993b). This Type I protein is membrane-anchored in the secretory granule with its two catalytic domains in the lumen of the granule and the cytosolic domain in the cytosol. The rat PAM gene can undergo splicing to produce several forms of PAM, with the production of end-products being tissue-specific (Ouafik et al., 1989; Stoffers et al., 1991; Eipper, et al., 1992b).

A two-step reaction results in the amidation of glycine-extended peptides (Figure 8). The first step in the reaction is catalyzed by PHM and results in the formation of a peptidyl-α-hydroxyglycine intermediate. This step requires two atoms of copper (Cu²⁺), molecular oxygen and ascorbate (Vitamin C). Of note is that it has been shown that the chelation of copper reduces the activity of PHM; other metals cannot initiate PHM activity (Eipper et al., 1993a). It also has been shown that one mole of ascorbic acid is enough to produce one mole of peptidylglycine product, and that PHM converts ascorbate into semidehydroascorbate within the lumen of secretory granules (Ingebresten et al., 1980; Murthy et al., 1987; Eipper and Mains, 1991; Hamman et al., 1998). The second step of the reaction utilizes the PAL domain of PAM to catalyze and convert the peptidyl-α-hydroxyglycine intermediate into the α-amidated product.
Figure 6: The Topology of the PAM Protein in a Secretory Granule.
This diagram depicts the topological organization of peptidylglycine α-amidating monooxygenase (PAM) when localized to a secretory granule. PAM-1 is comprised of peptidyl α-hydroxylating monooxygenase (PHM), Exon A, peptidyl α-hydroxyglycine α-amidating lyase (PAL), a transmembrane domain (TMD) and a cytoplasmic domain (CD). (Modified from Kulathila et al., 1994).
Figure 7: The α-Amidation Reaction of PAM. The first step in the reaction involves the catalysis of the COOH-terminal glycine-extended peptide by PHM with the release of semi-dehydroascorbate. This reaction requires copper, ascorbate and molecular oxygen. In the second step, the intermediate, peptidyl-α-hydroxyglycine, is catalyzed by PAL to an amidated peptide with the release of glyoxylate. This process requires zinc. (Modified from Kulathila et al., 1994).
with the release of glyoxylate (Milgram et al., 1997; Prigge et al., 2000). The PAL domain also contains a metal binding region and typically utilizes zinc atoms (Bell et al., 1997).

PAM, a cuproenzyme, to which MNK functionally contributes to the delivery of copper, is expressed in a variety of tissues. PAM mRNA and protein are highly expressed in the pituitary, heart, hypothalamus and adrenals with lower expression in brain cortex, kidney, pancreas and stomach in adult rats (Braas et al., 1989; Braas et al., 1992; El Meskini et al., 2000). In addition, PAM mRNA levels and activity are developmentally regulated in lung and heart tissues in rats (Ouafik et al., 1989; Guembe et al., 1999). Interestingly, Steveson and colleagues (2003) have shown that in Mo<sup>br</sup> mice, expression levels of PAM-1 and its cleavage products are similar to the levels observed in WT mice.

Even though MNK and PAM mRNA and protein are expressed in brain and endocrine tissues, few studies have examined the relationship between MNK and PAM expression during development. However, studies have documented that the availability of copper proteins, including Cox17 and Ctr1, and the activity of cuproenzymes, such as PAM, cytochrome c oxidase, and SOD, influence neuronal development, brain function, the generation of membranes, and the growth of an individual (Yoshimura et al., 1990; Gaucheru et al., 1993; Prohaska, 1995; Prohaska, 2000; Lee et al., 2001). Furthermore, it has been demonstrated that in WT mice, copper concentration and MNK expression increase with age; however, in the Mo<sup>br</sup> mice MNK expression decreases with age. Although studies have confirmed that both MNK and PAM are expressed at similar levels in young mice, these studies have not examined whether changes during development are tissue-specific or if PAM expression depends on that of MNK expression or vice versa (Steveson et al., 2003).
To study the routing and trafficking of both membrane and soluble proteins through the regulated secretory pathway, such as PAM, researchers transfected the pituitary adrenocorticotrope tumor cell line, AtT-20, in which MNK is endogenously expressed, with a PAM-1 construct (Milgram et al., 1992; Tausk et al., 1992; Maltese and Eipper, 1993; Milgram et al., 1993). It should be noted that AtT-20 cells possess characteristics similar to those of the pituitary corticotrope cells from which they are derived. When corticotropes are stimulated by the hypothalamic releasing hormone, CRH, they synthesize and package the precursor protein, proopiomelanocortin (POMC), into secretory vesicles, where it is further cleaved by prohormone convertases 1 (PC1) into adrenocorticotropic hormone (ACTH) for release from the cell (Aoki et al., 1997). CRH also influences the synthesis, processing and secretion of PAM in both AtT-20 and anterior pituitary cells (Thiele and Eipper 1990; Giraud et al., 1992; Ciccotoso et al., 1999). Of note is that AtT-20 cells have been documented to express lower levels of PAM and have fewer secretory granules than corticotrope cells. Furthermore, levels of POMC have been observed to be lower in AtT-20 cells than in corticotropes (Tooze, 1998; Ciccotosto et al., 1999).

PAM has become a useful model to examine the trafficking and localization of granular proteins within the secretory pathway as it found in both soluble and membrane forms (Tausk et al., 1992; Maltese and Eipper, 1993). Immunofluorescence microscopy and subcellular fractionation studies have determined that PAM is localized to the TGN region as well as to lighter compartments corresponding to secretory granules in anterior pituitary cells as well as in AtT-20 cells (Milgram et al., 1992; Milgram et al., 1993; Oyarce and Eipper, 1995; Meskini et al., 2000). PAM localization and secretion occur in a cell specific manner in each of the five cell types that compose the anterior pituitary (El Meskini et al., 2000). Furthermore, PAM undergoes processing to soluble PHM and membrane bound PAL in secretory granules, before regulated
exocytosis causes the release of PHM into the extracellular milieu in both corticopitropes and AtT-20 PAM-1 cells. Although membrane bound forms of PAM are present at the plasma membrane, there is little accumulation, as the membrane bound forms are taken up in endosomes from the cell surface and delivered to lysosomes, granules, or back to the TGN (Milgram et al., 1992; Milgram et al., 1993; El Meskini et al., 2001; Steveson et al., 2001). Interestingly, sequences in the cytosolic domain of PAM help route PAM within the secretory pathway (Yun et al., 1995; Steveson et al, 1999).

In addition, PAM and its cleavage products are localized to secretory granules along with other resident granular proteins, including PC1 and carboxypeptidase E (CPE) (Varlamov et al., 1998). CPE is involved in prohormone processing, while PC1 is involved in the cleavage of both POMC and PAM (Marx et al., 1999; Muller et al., 2000). Overall, these data indicate that PAM is localized in secretory granules along with other key granular proteins as well as with the proteins it modifies.

Having established that the localization of PAM varies in each of the cell types in the pituitary, PAM secretion in response to various stimulatory factors was examined. El Meskini and colleagues (2000) demonstrated that several hypothalamic-releasing hormones could influence the activity of PHM as well as the secretion of PAM from both the anterior pituitary and AtT-20 PAM-1 cells, including: GnRH, growth hormone releasing hormone (GHRH), CRH, and TRH (El Meskini et al., 2000). As expected, in response to these releasing hormones, the processing of PAM and secretion levels varied depending on the individual pituitary cell types. However, in each case, it was shown that the pituitary cells could be stimulated by external stimuli to undergo exocytosis; thus, PAM secretion from secretory granules in these cells is regulated.
Two general secretagogues, barium chloride (BaCl$_2$) and phorbol myristate acetate (PMA), also have been used to examine the exocytosis of secretory granules and their contents from pituitary and AtT-20 cells (Thiele and Eipper, 1990). In general, BaCl$_2$ acts like calcium to cause the exocytosis of mature secretory granules, while PMA activates a protein kinase C signaling pathway to cause the release of both immature and mature secretory granules (El Meskini $et$ $al.$, 2000). In AtT-20 and pituitary cells, BaCl$_2$ has been shown to stimulate the secretion of PAM from mature secretory granules as well as to increase PHM activity (El Meskini $et$ $al.$, 2000; Ferraro $et$ $al.$, 2005). Similar results were observed in anterior pituitary cells treated with PMA. Overall, these studies demonstrate that the release of PAM from secretory granules can be regulated by secretagogues. Interestingly, studies have not been done to elucidate whether the same regulators of PAM synthesis and secretion may regulate the synthesis of MNK. Since the addition of external stimuli alters the expression of PAM and since PAM receives copper from MNK, it is possible that MNK expression and localization could be altered or regulated by these same stimuli.

In the following dissertation, the first chapter focuses on evaluating the localization of the MNK protein in AtT-20 WT and PAM-1 cells in response to various copper levels by immunofluorescence microscopy and subcellular fractionation. A comparison of the localization of the MNK protein in WT and PAM-1 cells was made to determine if the localization of the MNK protein is altered by the presence of the PAM protein, since PAM relies on the MNK protein for the delivery of copper. The second chapter aims to characterize the expression of MNK mRNA and protein during rat development in brain and endocrine tissues. A comparison of MNK expression to that of the PAM protein also was performed to determine if there is a temporal correlation between the expression of MNK and PAM. Finally, the goal of the third
chapter is to examine if hypothalamic-releasing hormones and general secretagogues, known to cause exocytosis of secretory granules and increase expression of PAM, influence the localization and expression of the MNK protein in AtT-20 WT and PAM-1 cells. Overall, we hope these studies help us gain a better understanding of the expression, localization and regulation of MNK in endocrine cells.
Chapter 1
TRAFFICKING AND SUBCELLULAR LOCALIZATION OF THE MNK PROTEIN IN ENDOCRINE CELLS

Previous studies have examined the localization of the MNK protein in human fibroblasts and CHO cells transfected with the MNK protein (La Fontaine et al., 1998; Kim et al., 2003). Results from these studies have shown that the MNK protein is localized to the TGN at steady-state. In cells treated with high levels of copper, the MNK protein trafficks from the TGN towards the plasma membrane and when levels of copper are reduced, the MNK protein trafficks back towards the TGN (Dierik et al., 1997, Pascale et al., 2003). Few studies have aimed to examine the localization of the MNK protein in endocrine cells at steady-state and in response to various copper levels. In general, these studies have not fully examined the interactions of the MNK protein with other proteins, such as the cuproenzyme, PAM.

To determine the localization of the copper transporting protein MNK, and to compare the subcellular localization of the MNK protein to the amidating enzyme, PAM, cells derived from an adrenocorticotropic tumor cell line (AtT-20) were used for these studies. AtT-20 cells were chosen as they are readily available in the lab and relatively easy to grow and to transfect. Furthermore, AtT-20 cells have been well characterized and have a regulated secretory pathway, since they are derived from endocrine cells. Finally, AtT-20 cells were used as they endogenously express MNK.

For these studies, the AtT-20 cells, previously transfected with PAM-1, were used to begin to gain a better understanding of the subcellular localization of the MNK protein in relationship to the localization of PAM. This relationship is important as it has been previously...
demonstrated that the MNK protein delivers copper to PAM (Steveson et al., 2003). Previous studies have demonstrated by immunostaining and subcellular fractionation that membrane bound PAM is localized mainly in secretory granules, the TGN, and recycling compartments in the pituitary (Oyarce and Eipper, 1995; El Meskini et al., 2000). Although it is known that the MNK protein can deliver copper to PAM, it is not known where in the secretory pathway this occurs. Therefore, in examining the subcellular localization of both the MNK and PAM proteins, a better understanding of the relationship of these membrane bound proteins in endocrine cells can be determined.

To begin to elucidate the subcellular localization of MNK and PAM, AtT-20 WT and PAM-1 cells were analyzed by immunofluorescence microscopy at steady-state (controls). Using MNK and PHM antibodies as well as various organelle markers, the localization of the MNK protein was compared to that of PAM as well as to the localization of the organelle markers. Subcellular fractionation using differential and equilibrium sucrose density gradient centrifugation also was used to allow us to further identify the subcellular organelles containing the MNK protein, as well as to determine if the MNK protein is co-localized with the PAM protein. Analyses of the subcellular fractions were done by Western blot analyses. In addition, immunofluorescence microscopy and subcellular fractionation were performed using AtT-20 WT and PAM-1 cells incubated in the presence or absence of excess copper to begin to establish the localization of the MNK protein in response to changes in copper levels.
METHODS

Cells

An adrenocorticotropic tumor derived cell line, stably transfected with PAM-1 (AtT-20 PAM-1), was grown in Dulbecco’s Modified Eagle’s Medium (DMEM/F12/CO2; Mediatech Inc., Herdon, VA), supplemented with 10% v/v Fetal Clone III (FCS; HyClone Laboratories, Inc., Logan, UT), 10% Nu-Serum IV (Collaborative Research, Bedford, MA), 0.5mg/ml G418 sulfate (Mediatech Inc., Herdon, VA), and 5 ml Penicillin Streptomycin (10,000 units/ml; Invitrogen Corp., Carlsbad, CA). AtT-20 WT cells were grown in the same media and supplements, but without the G418 sulfate. Cells were passed each week.

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed to obtain a visual representation of where the MNK protein is localized in the cell at steady-state, as well as its localization in cells incubated in various levels of copper. AtT-20 WT or PAM-1 cells were cultured on four, 4-well slides coated with poly-L-lysine for four days prior to fixation (Figure 9A and C). On the third day, two slides were incubated in DMEM/F-12/CO2 medium with bathocuproinedisulfonic acid (BCS; 50 μM; Sigma, St. Louis, MO) for 16 h at 37 °C. On the fourth day, one slide previously incubated with BCS, was then incubated with copper chloride (CuCl2; 200 μM; Sigma) for 5 h at 37 °C. On the fourth day, another slide was incubated in CuCl2 (200 μM) for 5 h at 37 °C. The untreated slide served as the control. Following treatments of the cells on all the slides, the cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) for 1 h and rinsed with
Figure 9: AtT-20 WT or PAM-1 Cells Incubated in the Presence or Absence of Copper. A. Set-up for the four well chamber slides for immunofluorescence microscopy experiments. B. Set-up for the plates for the subcellular fractionation experiments. Slide B and Plate F received CuCl₂ (200 µM), slide C and plate G received BCS (50 µM), slide D and plate H received both BCS/CuCl₂ and slide A and plate E were untreated. C. Treatment of plates and slides: cells were plated at time 0 in 100 mm dishes (E, F, G, and H) or in chamber slides (A, B, C, and D). A and E were not treated during the 72 h and served as a control. B and F were treated at 67 h with CuCl₂. C and G were treated with BCS at 56 h. D and H were treated with BCS at 51 h, and at 67 h, BCS was removed, and CuCl₂ was added. Slides A, B, C, and D were fixed at 72 h for immunofluorescence microscopy. Plates E, F, G, and H were extracted at 72 h, homogenized and subjected to subcellular fractionation.
PBS over 30 min. All cells were permeabilized with 0.1% Triton X-100 in PBS for 20 min and incubated in 2 mg/ml bovine serum albumin (BSA) in PBS to block non-specific binding of antibody. Cells then were incubated with either primary mouse monoclonal or polyclonal antibodies (see Table 2), diluted in 2 mg/ml BSA solution, and stored overnight at 4 °C. The primary polyclonal antibodies and respective concentrations used for these studies were as follows: carboxyl-terminal specific MNK CT77 [amino acids (a.a.) 1475-1492, 1:500], PHM JH1764 (a.a. 37-382, 1:1000), TGN38 JH1479 (a.a. 155-249, 1:500) and PC1 JH888 (a.a. 359-373, 1:1000) (Zhou and Mains, 1994; Milgram et al., 1997; Steveson et al., 1999; Steveson et al., 2003). The primary monoclonal antibodies used for these studies were: a clathrin coated pit marker (α-adaptin; 1:500; BD Bioscience, San Jose, CA), a plasma membrane marker, soluble N-ethyl-maleimide-sensitive factor attachment protein (SNAP-25; 1:500; BD Bioscience), and a secretory granule marker (chromogranin A; CGA; 1:1500; BD Bioscience). The following day, the primary antibodies were removed and the cells rinsed with PBS for 30 min. Cells incubated with the monoclonal antibodies were then incubated with a Cy3-conjugated donkey antibody to mouse IgG (Cy3-DAM; 1:1000; Jackson Immunoresearch Laboratories Inc., West Grove, PA), whereas the cells incubated with the polyclonal antibodies were treated with fluorescein isothiocyanate-conjugated goat antibody to rabbit IgG (GAR-FITC; 1:1000; Caltag Laboratories, Burlingame, CA). After incubation with the secondary antibodies, the cells were rinsed with PBS over 30 min. A drop of permaflour (Beckman Coulter Inc., Fullerton, CA) containing 1,4-diazabicyclo (2.2.2.) octane (Sigma) was added to the slide to prevent fading and a cover slip was placed on top. Cells were examined using epifluorescence optics on a Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY) and photographed with a Photometric PVCAM Nikon
Table 2: List of Antibodies Used for Western Blot Analyses and Immunofluorescence Microscopy. These antibodies were used to help confirm the localization of MNK in AtT-20 cells.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Molecular Mass Detected (kDa)</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNK CT77</td>
<td>Rabbit IgG</td>
<td>178</td>
<td>Various</td>
</tr>
<tr>
<td>PHM JH1764</td>
<td>Rabbit IgG</td>
<td>120, 70, &amp; 45</td>
<td>TGN &amp; Secretory Granules</td>
</tr>
<tr>
<td>Prohormone Convertase 1 (PC1) JH888</td>
<td>Rabbit IgG</td>
<td>80 &amp; 60</td>
<td>Secretory Granules</td>
</tr>
<tr>
<td>TGN38 JH1479</td>
<td>Rabbit IgG</td>
<td>80</td>
<td>TGN</td>
</tr>
<tr>
<td>α-Adaptin</td>
<td>Mouse IgG</td>
<td>112</td>
<td>Clathrin Coated Pits</td>
</tr>
<tr>
<td>Chromogranin A (CGA)</td>
<td>Mouse IgG</td>
<td>86</td>
<td>Secretory Granules</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Mouse IgG</td>
<td>25</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>Synaptotagmin (p65)</td>
<td>Mouse IgG</td>
<td>65</td>
<td>Vesicles</td>
</tr>
</tbody>
</table>

**Antibody**: These antibodies were used to help confirm the localization of MNK in AtT-20 cells.
Eclipse E800 camera (Roper Scientific, Trenton, NJ) aided by Dr. Marilyn Cayer in the Microscopy Facility in the Biology Department of Bowling Green State University, Bowling Green, OH (Steveson et al., 2003; El Meskini et al., 2000).

**Subcellular Fractionation**

To further determine the subcellular localization of the MNK protein in the AtT-20 WT and PAM-1 cells at steady-state and following exposure to various copper levels, subcellular fractionation followed by Western blot analyses was performed. Either AtT-20 WT or PAM-1 cells were plated on 8 x 100 mm dishes and grown in DMEM/F-12/CO2 medium with supplements. After four days of growth, cells were treated with BCS and CuCl2 as described (Fig. 9B and C). Following treatment, cells from two dishes treated the same way were combined, centrifuged and re-suspended in homogenizing buffer (0.32 M Sucrose, 10 mM Tris-HCl, pH 7.4) containing protease inhibitors [40 µg/ml phenylmethylsulfonyl fluoride (PMSF; Sigma) and X (10 µg/ml lima bean trypsin inhibitor, 2 µg/ml leupeptin, 16 µg/ml benzamidine, and 2 µg/ml pepstatin; Sigma]. Cells were passed through a 26-gauge needle six times to break up clumps of cells and then homogenized in a ball-bearing cell homogenizer on ice using 12 strokes to break open the cells. Homogenates were centrifuged using a TL100.4 fixed angle rotor ultracentrifuge in a TL-100 ultracentrifuge (Beckman Coulter Inc., Fullerton, CA) with the assistance of Dr. Ana Maria Oyarce in the Department of Pharmacology, Cardiovascular Biology and Metabolic Disease, at the Medical University of Ohio, Toledo, OH. For differential centrifugation, samples were centrifuged at 5,000 rpm for 5 min to obtain a pellet (P1; nuclear fraction) and supernatant (S1) (Figure 10). S1 was further centrifuged at 10,000 rpm for 15 min to obtain the P2 pellet, enriched in mitochondria, the TGN, and secretory granules, and S2.
Figure 10: Samples Obtained by Differential Centrifugation.

AtT-20 WT or PAM-1 cells were grown for four days prior to fractionation and treatment. Untreated or treated cells were homogenized and spun in a TL-100 ultracentrifuge for 5 min at 5,000 rpm to obtain pellet 1 (P1) and supernatant 1 (S1). S1 was centrifuged further for 15 min at 10,000 rpm to obtain a pellet 2 (P2) and supernatant 2 (S2). S2 was centrifuged for 15 min at 30,000 rpm to obtain a pellet 3 (P3) and supernatant 3 (S3). Finally, S3 was centrifuged for 15 min at 100,000 rpm to obtain pellet 4 (P4) and supernatant 4, also called the cytosolic fraction. Pellet (P), supernatant (S).
S2 was centrifuged at 30,000 rpm for 15 min to obtain the P3 pellet, enriched in secretory
granules and smaller vesicles, and S3. S3 was centrifuged further at 100,000 rpm for 15 min to
obtain the P4, enriched in microsomes and S4 containing cytosol (Oyarce and Eipper, 1995; El
Meskini et al., 2001).

For further fractionation using sucrose density gradients, the P2 and P3 pellets were re-
suspended individually in 250 µl of 0.32 M sucrose solution with protease inhibitors (1:100
PMSF and X) and kept on ice. Aliquots of 180 µl each of P2 and P3 suspension were layered
onto two separate sucrose density gradients containing from the bottom to top: 1.6 M sucrose
(200 µl), 1.4 M sucrose (350 µl), 1.2 M sucrose (350 µl), 1.0 M sucrose (350 µl), 0.8 M sucrose
(350 µl), 0.6 M sucrose (250 µl), and 0.4 M sucrose (200 µl) with protease inhibitors (Figure 11).
The gradients were centrifuged in a swinging bucket TLS-55 rotor in a Beckman ultracentrifuge
for 2 h at 55,000 rpm, and fourteen 157 µl fractions were collected from the top to the bottom of
each gradient. Fractions were stored at –80°C for further examination by Western blot analyses.
The subcellular fractionation experiments were repeated a total of three times for each control
and treated set.

**Western Blot Analyses**

To analyze the distribution of the MNK protein in the subcellular fractions obtained by
differential and equilibrium centrifugation, Western blot analyses were performed. Aliquots of
30 µl of each sucrose density fraction and rainbow molecular mass standards (GE Healthcare,
Piscataway, NJ) were run at the same time to establish the apparent molecular masses of the
proteins on either 10 % or 12 % polyacrylamide, 0.25 % N,N’-methylene-bis-
P2 and P3 pellets each re-suspended in 250 µl of a 0.32 M Sucrose, 10 mM Tris (pH 7.4) solution with protease inhibitors.

Figure 11. Sucrose Density Gradients for Equilibrium Gradient Centrifugation of P2 and P3. 180 µl of re-suspended sample from P2 or P3 was layered on top of each gradient and centrifuged at 55,000 x g for 2 h. Fractions of 157 µl each were taken from the top of the gradient to the bottom. The top fraction was labeled fraction 1 and the bottom fraction, 14.
acrylamide/sodium dodecyl sulfate gels (SDS-polyacrylamide gel electrophoresis; SDS-PAGE). Aliquots with the same percentage of the total resuspension of the homogenates (H), P2, P3, P4 and cytosol also were taken and fractionated by SDS-PAGE. Proteins were transferred to Immobilon-P-membranes (Millipore, Bedford, MA) for 3 h, and incubated in 5 % powdered milk reconstituted in 50 mM Tris-HCL, 150 mM NaCl, pH 7.5 (Fisher Chemical, St. Louis, MO) containing 0.05% Tween-20 (Sigma) (TTBS) for 1 h to prevent non-specific binding (Oyarce and Eipper, 1995). Following this blocking step, the membranes were incubated either with one of the rabbit polyclonal antisera, MNK CT77 (1:1000), PHM JH1764 (1:1000), or PC1 JH888 (1:1000), or with one of the following mouse monoclonal antisera, SNAP-25 (1:500), α-adaptin (1:500), or CGA (1:250), overnight at 4 °C or at room temperature for 2 h. After incubation with the primary antibodies, the membranes were rinsed six times with TTBS over 30 min and incubated with donkey anti-rabbit IgG horseradish peroxidase-linked whole antibody (1:10,000; GE Healthcare) for the polyclonal primary antibodies or sheep anti-mouse IgG horseradish peroxidase-linked whole antibody (1:5000; GE Healthcare) for the monoclonal antibodies for 1 h at room temperature (RT). Membranes were rinsed three times over 15 min with TTBS, followed by one rinse with Tris buffered saline, and were visualized using the Amersham Enhanced Chemiluminescence kit (ECL; GE Healthcare). The blots were exposed to film for 30 sec to 30 min, depending on the antibody.
RESULTS

To first determine the localization of the MNK protein at steady-state, immunofluorescence microscopy of the AtT-20 WT cells was performed using the carboxyl-terminal specific MNK antibody and its localization pattern was compared to cells immunostained with antibodies to specific organelle markers. Immunofluorescence microscopy of the AtT-20 WT cells at steady-state immunostained with the MNK antibody showed MNK protein localization in the perinuclear region (Figure 12A; wide arrow) as well as diffuse staining in the cytoplasm of the cells (Fig. 12A; asterisk). This staining pattern of MNK suggests that MNK is localized to more than one subcellular compartment in AtT-20 WT cells.

To better characterize the localization of the MNK protein in the AtT-20 WT cells, organelle markers were used. AtT-20 WT cells at steady-state were immunostained and visualized using the following organelle markers: TGN 38/TGN, CGA/secretory granules, SNAP-25/plasma membrane, and α-adaptin/clathrin coated pits. Visualization of the TGN38 antibody showed perinuclear staining in the AtT-20 WT cells that resembled that of MNK protein staining observed in the perinuclear region (Fig. 12A; wide arrow). Visualization of the CGA antibody showed a diffuse granular staining (Fig. 12D) that was similar to the diffuse MNK protein staining observed in Fig. 12A (asterisk). Cells immunostained with the SNAP-25 antibody showed staining that was localized to the plasma membrane (Fig. 12E; double thin arrows) and immunostaining with the α-adaptin marker presented a diffuse staining suggestive of clathrin coated pits (Fig. 12C; single thin arrow). The staining pattern of the MNK protein in the AtT-20 WT cells (Fig. 12A) was similar to the staining patterns produced by the CGA and TGN antibodies (Fig. 12D), but differed from those produced by the α-adaptin (Fig. 12C) and SNAP-25 antibodies (Fig. 12E). Thus, the similarities to the MNK protein staining observed
Figure 12: Immunofluorescence Microscopy of the Localization of MNK and Organelle Markers at Steady-State in AtT-20 WT Cells. AtT-20 WT cells were grown on four well chamber slides and fixed in 4% paraformaldehyde. Cells were immunostained with either (A) the MNK antibody (1:1000), or (B) the TGN38 antibody (1:500) and then incubated with the FITC-conjugated rabbit IgG antibody. Cells were also immunostained with (C) the α-Adaptin antibody (1:500), (D) the CGA antibody (1:1000), or (E) the SNAP-25 antibody (1:500), and then incubated with a Cy3-conjugated mouse IgG. All cells were observed by epifluorescence microscopy. The scale bar equals 10 µm (panel E). Nucleus (N), wide arrows (TGN/perinuclear region), thin arrow (clathrin coated pits), double thin arrows (plasma membrane) and asterisk (vesicles).
using the TGN38 and CGA antibodies suggest that MNK is localized to the TGN and secretory granules in AtT-20 WT cells.

AtT-20 WT cells were cultured in varying concentrations of copper and immunostained with the MNK antibody to examine the effects of various copper levels on the localization of the MNK protein. In control AtT-20 WT cells, the MNK protein was predominantly localized in the perinuclear region (Figure 13A; wide arrow). When the AtT-20 WT cells were exposed to the copper chelator, BCS (50 µM), for 15 h before fixation, MNK protein localization again was observed in the perinuclear region (Fig. 13B; wide arrow). However, when the AtT-20 WT cells were cultured in high concentrations of copper (200 µM for 5 h), the MNK protein was localized in the perinuclear region as well as in some punctate vesicular structures distributed throughout the cytoplasm and potentially at the plasma membrane (Fig. 13C; arrowhead). In the AtT-20 WT cells incubated with BCS followed by copper, the MNK protein appeared to be localized to the perinuclear region with the same diffuse cytoplasmic staining pattern observed at steady-state (Fig. 13D; wide arrows). The localization of the MNK protein did not appear to be drastically different between treatments with BCS or BCS and copper; however, the treatment of copper alone did appear to alter the localization of the MNK protein.

To confirm the localization of the MNK protein in these treated AtT-20 WT cells, the cells were treated with BCS (50 µM) for 15 h, copper (200 µM) for 5 h or both BCS and copper (50 µM for 15 h and 200 µM for 5 h) and then immunostained with various organelle markers (TGN38, PC1, α-adaptin, and SNAP-25). Regardless of the treatments, the AtT-20 WT cells when immunostained with the TGN38 antibody presented with intense staining in the perinuclear region, corresponding to the Golgi/TGN (Figure 14A, B, C, and D). Furthermore, untreated control and treated AtT-20 WT cells immunostained with the PC1 antibody, a secretory granule
Figure 13: Immunofluorescence Microscopy of the MNK Protein in AtT-20 WT Cells at Steady-State and Following Exposure to High and Low Levels of Copper. AtT-20 WT cells were treated as described, immunostained with the MNK antibody (1:500) and a FITC-conjugated rabbit IgG antibody. All cells were observed by epifluorescence microscopy. The scale bar equals 10 µm (Panel D). Nucleus (N), wide arrows (TGN/perinuclear region) and arrowhead (secretory granules).
Figure 14: Immunofluorescence Microscopy of TGN in AtT-20 WT Cells at Steady-State and Following Exposure to High and Low Levels of Copper. AtT-20 WT cells were treated, immunostained with the TGN38 antibody (1:500), and then incubated with a FITC-conjugated rabbit IgG antibody. All cells were observed by epifluorescence microscopy. The scale bar equals 10 µm (Panel D). Nucleus (N) and wide arrows (TGN/perinuclear region).
marker, showed a more diffuse punctate staining pattern with staining out in the tips of the cells, where secretory granules are located (Figure 15A, B, C, and D; arrowheads).

AtT-20 WT cells treated and then immunostained with the α-adaptin antibody showed a punctate staining pattern throughout the cytoplasm, indicating the presence of clathrin coated vesicles (Figure 16A, B, C, and D; single thin arrows); control untreated and treated cell staining was similar. Finally, treated AtT-20 WT cells immunostained with the SNAP-25 antibody showed staining of the plasma membrane at the outer edges of the cells, which again did not vary between the control untreated and treated cells (Figure 17A, B, C, and D; double thin arrows). Therefore, the comparison of the MNK protein staining pattern to that of the organelle markers in the AtT-20 WT cells, indicates that the MNK protein was localized primarily to the TGN and secretory granules at steady-state as well as in cells treated with BCS or BCS and copper, while trafficking of the MNK protein, perhaps on its way to the plasma membrane, was observed in cells treated with copper.

To further determine the localization of the MNK protein, AtT-20 cells previously transfected with the cuproenzyme, PAM-1, also were used. Although there is evidence to suggest that the MNK protein delivers copper to the cuproenzyme, PAM, where this occurs in the regulated secretory pathway has yet to be elucidated. Immunofluorescence microscopy analyses were performed to determine if of the MNK and PAM proteins are co-localized in AtT-20 cells stably transfected with PAM-1.

For these studies, AtT-20 PAM-1 cells were immunostained with the MNK antibody and the organelle markers (PHM, PC1, TGN38, SNAP-25, and α-adaptin) as described in the methods. In the AtT-20 PAM-1 cells at steady-state, MNK protein staining (Figure 18A) was observed in the perinuclear region resembling the TGN38 staining (Fig. 18C) as well as in
Figure 15: Immunofluorescence Microscopy of PC1 in AtT-20 WT Cells at Steady-State and Following Exposure to High and Low Levels of Copper. AtT-20 WT cells were treated as described, immunostained with the PC1 888 antibody (1:1000) and then incubated with a FITC-conjugated rabbit IgG antibody. All cells were observed by epifluorescence microscopy. The scale bar equals 10 μm (Panel D). Nucleus (N) and arrowheads (secretory granules).
Figure 16: Immunofluorescence Microscopy of α-Adaptin in AtT-20 WT Cells at Steady-State and Following Exposure to High and Low Levels of Copper. AtT-20 WT cells were treated as described, immunostained with the α-adaptin antibody (1:500), and then incubated with a Cy3-conjugated mouse IgG. All cells were observed by epifluorescence microscopy. The scale bar equals 10 µm (Panel D). Nucleus (N) and single thin arrows (clathrin coated pits).
Figure 17: Immunofluorescence Microscopy of SNAP-25 in AtT-20 WT Cells at Steady-State and Following Exposure to High and Low Levels of Copper. AtT-20 WT cells were treated as described, immunostained with the SNAP-25 antibody (1:500), and then incubated with a Cy3-conjugated mouse IgG. All cells were observed by epifluorescence microscopy. The scale bar equals 10 µm (Panel D). Nucleus (N) and double thin arrows (plasma membrane).
Figure 18: Immunofluorescence Microscopy of the Localization of the MNK and PAM Proteins and Organelle Markers at Steady-State in AtT-20 PAM-1 Cells. Untreated (control) AtT-20 PAM-1 cells were grown on four well chamber slides and fixed in 4% paraformaldehyde. Cells were immunostained with (A) the MNK antibody (1:1000), (B) the PHM antibody (1:1000), or (C) the TGN38 antibody (1:500) and then incubated with FITC-conjugated rabbit IgG antibody. Cells also were immunostained with (D) the CGA antibody (1:1000), (E) the SNAP-25 antibody (1:500), or (F) the α-Adaptin antibody (1:500) and then incubated with a Cy3-conjugated mouse IgG. All cells were observed by epifluorescence microscopy. The scale bar equals 10 µm (Panel F). Nucleus (N), wide arrows (TGN/perinuclear region), arrowheads (secretory granules), single thin arrow (clathrin coated pits), and double thin arrows (plasma membrane).
punctate structures, reminiscent of the staining observed with the secretory granule marker, CGA (Fig. 18D) and α-adaptin, a clathrin coated pit marker (Fig. 18F). No staining was observed at the plasma membrane as compared to SNAP-25 (Fig. 18E). In the AtT-20 PAM-1 cells immunostained with the PHM antibody, the PAM protein was detected primarily in the perinuclear region (Fig. 18B) with some staining in the secretory granules, as observed with the MNK antibody (Fig. 18A). The staining patterns for the various markers (TGN38, CGA, SNAP-25, and α-adaptin) (Fig. 18C, D, E, and F) in the AtT-20 PAM-1 cells were similar to those observed in the AtT-20 WT cells (Fig. 12B, C, D, and E). These results indicate that in AtT-20 PAM-1 cells at steady-state, the MNK protein co-localizes with the PAM protein in the TGN region as well as in secretory granules.

To further examine the effects of copper levels on the localization of the MNK protein in the At-T-20 PAM-1 cells, the cells were treated with the copper chelator, BCS (50 µM for 15 h), or copper (200 µM for 5 h), or both BCS and copper (50 µM for 15 h and 200 µM for 5h) followed by immunofluorescence microscopy as described in the methods. In the control AtT-20 PAM-1 cells immunostained with the MNK antibody, the MNK protein was localized to the perinuclear region with diffuse staining in the cytoplasm (Figure 19A; wide arrow). When the AtT-20 PAM-1 cells were exposed to BCS, MNK protein staining was observed in a more tightly compact perinuclear region (Fig. 19B; wide arrow) than in the control cells. Following incubation of the AtT-20 PAM-1 with either copper or BCS and copper, MNK staining was observed in the perinuclear region, with more diffuse punctuate staining observed throughout the cytoplasm (Fig. 19C and D, asterisks).

AtT-20 PAM-1 cells treated with BCS, copper, or BCS and copper were immunostained with the PHM antibody to determine localization of the PAM and PHM proteins. PAM and
Figure 19: Immunofluorescence Microscopy of the MNK Protein in AtT-20 PAM-1 Cells at Steady-State and Following Exposure to High and Low Levels of Copper. AtT-20 PAM-1 cells were treated as described, fixed in 4% paraformaldehyde and immunostained with the MNK antibody (1:1000) and a FITC-conjugated rabbit IgG. All cells were observed by epifluorescence microscopy. The scale bar equals 10 µm (Panel D). Nucleus (N), wide arrows (TGN/perinuclear region) and asterisks (cytoplasmic staining).
Figure 20: Immunofluorescence Microscopy of the PHM Protein in AtT-20 PAM-1 cells at Steady-State and Following Exposure to High and Low Levels of Copper. AtT-20 PAM-1 cells were treated as described, immunostained with the PHM antibody (1:1000) and a FITC-conjugated rabbit IgG antibody. All cells were observed by epifluorescence microscopy. The scale bar equals 10 µm (Panel D). Nucleus (N), wide arrows (TGN/perinuclear region) and arrowhead (secretory granules).
PHM immunostaining was observed mainly in the TGN and the tips of the processes (Figure 20A, B, C, and D; wide arrows and arrowheads). Furthermore, the staining pattern of the PAM and PHM proteins did not change in response to copper levels. As before, the staining pattern of the treated cells immunostained with the MNK antibody did appear to be similar to the staining patterns observed for the PAM and PHM proteins.

To analyze the effects of copper levels on the localization of organelle markers, AtT-20 PAM-1 cells were treated and immunostained with the following antibodies: PHM, TGN38, CGA, PC1, SNAP-25 and α-adaptin. As with the AtT-20 WT cells, regardless of treatment in the AtT-20 PAM-1 cells, the markers immunostained the appropriate organelles. In the control and treated AtT-20 PAM-1 cells, TGN38 was localized to the perinuclear region (Figure 21A, B, C, and D; wide arrows), and PC1 showed a diffuse staining pattern throughout the cytoplasm, concentrating in the tips of the cells (Figure 22A, B, C, and D; asterisks and arrowheads). In addition, SNAP-25 staining was observed at the plasma membrane (Figure 23A, B, C, and D; double thin arrows), and lastly, α-adaptin staining was observed as a diffuse punctate staining pattern indicating the presence of clathrin coated pits (Figure 24A, B, C, and D; single thin arrows).

To further examine the localization of the MNK protein in the AtT-20 WT and PAM-1 cells and to confirm the results from the immunostaining experiments, subcellular fractionation and Western blot analyses were performed as described in the methods. The initial pellets obtained by differential centrifugation were fractionated by SDS-PAGE and visualized with the MNK antibody and organelle markers (Figure 25). In the AtT-20 WT cells, the MNK protein was detected in the homogenate (H) and pelleted fractions (P2-4) as a 178 kDa band (Fig. 25; 1st
Figure 21: Immunofluorescence Microscopy of TGN in AtT-20 PAM-1 Cells at Steady-State and Following Exposure to High and Low Levels of Copper. AtT-20 PAM-1 cells were treated as described, immunostained with the TGN38 antibody (1:500) and a FITC-conjugated rabbit IgG antibody. All cells were observed by epifluorescence microscopy. Scale bar equals 10 µm (Panel D). Nucleus (N) and wide arrows (TGN/perinuclear region).
Figure 22: Immunofluorescence Microscopy of PC1 in AtT-20 PAM-1 Cells at Steady-State and Following Exposure to High and Low Levels of Copper. AtT-20 PAM-1 cells were treated as described, immunostained with the PC1 antibody (1:1000), and then incubated with FITC-conjugated rabbit IgG antibody. All cells were observed by epifluorescence microscopy. The scale bar equals 10µm (Panel D). Nucleus (N), arrowheads (secretory granules) and asterisks (cytoplasmic staining).
Figure 23: Immunofluorescence Microscopy of SNAP-25 in AtT-20 PAM-1 Cells at Steady-State and Following Exposure to High and Low Levels of Copper. Cells were treated as described, immunostained with the SNAP-25 antibody (1:500) and a Cy3-conjugated mouse IgG. All cells were observed by epifluorescence microscopy. The scale bar equals 10 µm (Panel D). Nucleus(N), double thin arrows (plasma membrane).
Figure 24: Immunofluorescence Microscopy of α-Adaptin in AtT-20 PAM-1 Cells at Steady-State and Following Exposure to High and Low Levels of Copper. Cells were treated as described, immunostained with the α-adaptin antibody (1:1000) and a Cy3-conjugated mouse IgG. All cells were observed by epifluorescence microscopy and pictures were taken at a magnification of 40x. The scale bar equals 10 µm (Panel D). Nucleus (N) and single thin arrows (clathrin coated pits).
Figure 25: Western Blot Analyses Following Differential Centrifugation of AtT-20 WT Cells at Steady-State. AtT-20 WT cells were cultured for four days prior to the experiment and differential centrifugation was performed as described. Sample H was 1% of the total suspension, the P2 and P3 samples were 21% of the total suspension. P4 and C were 6% and 2%, respectively, of the total suspension. Samples were resolved on 10 % gels, visualized with the MNK, α-adaptin, TGN38, PC1, and SNAP-25 antibodies, using the ECL detection system as described in the methods. Non-specific binding of antibody (asterisks). Lanes with less staining (plus sign).
A non-specific band above the MNK band was detected in P4 (asterisk), and two non-specific bands were observed in the cytosol (Fig. 25; 1st panel; C; asterisks). A clathrin coated pit specific marker, α-adaptin, detected a single band at 112 kDa in H, P2-P4 and C, with an enrichment in P2 and P3 (Fig. 25; 2nd panel). The TGN38 antibody detected bands at 80 kDa in H, P2-P4. A non-specific band was detected in the C fraction (Fig. 25; 3rd panel; asterisk). PC1, a secretory granule protein was observed in H, P2 and P3 as two bands of 80 and 60 kDa, respectively (Fig. 25; 4th panel). A previous study has shown that beginning in the TGN, the PC1 protein is 80 kDa and as it traffics into the secretory granules, cleavage occurs resulting in an 60 kDa form of PC1, which is the more dominant form of PC1 stored in secretory granules (Zhou and Mains, 1994). SNAP-25, a plasma membrane protein, was detected as a 25 kDa band in the P2 and P3, with enrichment in P2 (Fig. 25; 5th panel). These results taken together indicate that the MNK protein is localized to the same regions as that of the TGN, secretory granules, clathrin coated pits and perhaps the plasma membrane (Fig. 25).

The distribution of the MNK protein and the organelle markers in the initial pellets obtained by the differential centrifugation for the AtT-20 PAM-1 cells was determined by Western blot analyses using the same antibodies described above (Figure 26). In the AtT-20 PAM-1 cells, the MNK protein was detected in H, P2 and P3 as a 178 kDa band (Fig. 26; 1st panel), while no detectable MNK was observed in P4 or the cytosol. In the AtT-20 PAM-1 cells, the PHM antibody detected the full length PAM protein and its processed products, soluble PAM-1 (105 kDa) and PHM (45 kDa), primarily in the H and the P2 and P3 pellets, with no significant amounts detected in P4 or the cytosol (Fig. 26; 2nd panel). The clathrin coated pit marker, α-adaptin, was detected as a single band of 112 kDa in H and C, and in the P2-P4 pellets (Fig. 26; 3rd panel). The TGN38 antibody detected the TGN protein at 80 kDa in the H and the
Figure 26: Western Blot Analyses Following Differential Centrifugation of AtT-20 PAM-1 cells. AtT-20 PAM-1 cells were cultured for four days prior to the experiment and differential centrifugation was performed as described. Sample H was 1% of the total suspension, the P2 and P3 samples were 21% of the total suspension. P4 and C were 6% and 2%, respectively, of the total suspension. Samples were resolved on 10% gels and visualized with MNK, PHM, PC1, SNAP-25 and α-adaptin antibodies as described in the methods.
The secretory granule protein, PC1, was detected in H, P2 and P3 as two bands at 80 and 60 kDa, as observed in AtT-20 WT cells (Fig. 26; 5th panel). Finally, SNAP-25, a plasma membrane protein, was detected as a 25 kDa band in H and the P2 and P3 pellets (Fig 26; 6th panel). These results taken together indicate that the MNK protein was enriched in the same pelleted fractions that overlap the TGN, secretory granules, clathrin coated pits and the plasma membrane (Fig 26), again suggesting that the MNK protein is localized to more than one subcellular compartment in AtT-20 cells.

To further analyze the subcellular distribution of the MNK protein in different copper levels, differential centrifugation was performed following the treatment of the AtT-20 WT and PAM-1 cells with BCS (50 µM; 16 h), copper (200 µM; 5 h), or BCS and copper. The subcellular fractions were resolved by SDS-PAGE and visualized using the MNK antibody (Figure 27). In both cells types, regardless of treatment, the MNK protein was mainly observed in H and the P2 and P3 pellets. In the AtT-20 WT type cells, the two bands observed in the cytosol are non-specific (Fig. 27; 1st panel, asterisks). Interestingly, in the control and AtT-20 WT cells treated with BCS, significantly more MNK protein was observed in the P4 pellet than from the cells exposed to copper or BCS/copper (Fig. 27; 2nd panel). Overall, these results demonstrate that the distribution of the MNK protein in the differential centrifugation fractions is similar in both AtT-20 WT and PAM-1 control and treated cells.

To further analyze the localization of the MNK protein, Western blot analyses were performed on AtT-20 PAM-1 cells following differential centrifugation using the PHM antibody (Figure 28). In the control AtT-20 PAM-1 cells, the PAM-1 (120 kDa) and PHM (45 kDa) proteins were detected in H, P2 and P3, with faint expression in the cytosol (C) (Fig. 28; top left panel). In the AtT-20 PAM-1 cells treated with BCS, copper or BCS/copper, the PAM-1, sPAM
### AtT-20 Wild Type Cells

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### AtT-20 PAM-1 Cells

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**Figure 27: Western Blot Analyses of the MNK Protein Following Differential Centrifugation in AtT-20 WT and PAM-1 Cells Exposed to Various Copper Levels.** AtT-20 WT and PAM-1 cells were cultured for four days prior to the experiment, treated as described, and differential centrifugation was performed. Sample H was 1% of the total suspension, the P2-P4 samples were 21% of the total suspension, and sample C was 2% of the total suspension. Samples were resolved on 10% gels and visualized with MNK antibody as described in the methods. Non-specific bands (*asterisks*).
Figure 28: Western Blot Analyses of the PAM Protein Following Differential Centrifugation in AtT-20 PAM-1 Cells Exposed to Various Copper Levels. AtT-20 PAM-1 cells were cultured for four days prior to the experiment, treated as described, and differential centrifugation was performed. Sample H was 1% of the total suspension, and the P2 and P3 samples were 21% of the total suspension. P4 and C were 6% and 2%, respectively, of the total suspension. Samples were resolved on 10% gels and visualized with the PHM antibody as described in the methods.
(105 kDa), and PHM proteins showed a similar distribution to that of the control cells in the H, P2-P4 (Fig. 28; top right, bottom left and right panels). The presence of the PHM protein (45 kDa) indicates the presence of secretory granules in these fractions.

The initial pellets obtained following differential centrifugation of untreated and treated AtT-20 WT and PAM-1 cells were analyzed further to determine the presence of secretory granules by Western blot analyses using the PC1 antibody. The PC1 antibody detected bands at 80 and 60 kDa, respectively, in both the AtT-20 WT (Figure 29; left panels) and PAM-1 cells (Fig. 29; right panels). In both the control and BCS treated AtT-20 WT cells, 80 kDa PC1 was observed in the H, P2 and P3 samples, while the 60 kDa band was only faintly observed in the same samples. In the cells treated with copper and BCS/copper, the 60 kDa band was detected in H, P2 and P3, and faintly in the cytosol (C). However, in the BCS/copper treated cells, the 80 kDa PC1 band was detected in P2 and P3 and in the copper treated cells, the 80 kDa band was faintly observed in H and P3, with a stronger intensity of band observed in P2. In the AtT-20 PAM-1 control, BCS, copper and BCS/copper cells, the 60 and 80 kDa PC1 bands were observed in the H, P2 and P3. In addition, in the copper treated cells, PC1 was also observed in the P4 sample. These results from both the AtT-20 WT and PAM-1 control and treated cells suggest that the P2 and P3 pelleted samples are enriched in secretory granules. Moreover, the treatments did not change the localization of these granules.

Overall, the SNAP-25 antibody, a plasma membrane marker, detected a single band at 25 kDa. In the control, BCS, and BCS/copper treated AtT-20 WT cells, SNAP-25 was observed in the H, P2-P4 pellets (Figure 30; left panels), while in the cells treated with copper, SNAP-25 was not detected in the P4 sample (Fig. 30; left panel). In the At-T 20 PAM-1 untreated and treated cells, SNAP-25 was observed in H, P2, P3 and slightly in the P4 samples (Fig. 30; right panels).
AtT-20 Wild Type Cells

Control

AtT-20 PAM-1 Cells

Control

H  P2 P3 P4 C

BCS

H  P2 P3 P4 C

CuCl₂

H  P2 P3 P4 C

BCS/CuCl₂

H  P2 P3 P4 C

Figure 29: Western Blot Analyses of PC1 Following Differential Centrifugation in AtT-20 WT and PAM-1 Cells. AtT-20 WT and PAM-1 cells were cultured for four days prior to the experiment, treated as described, and differential centrifugation was performed. Sample H was 1% of the total suspension and the P2 and P3 samples were 21% of the total suspension. P4 and C were 6% and 2%, respectively, of the total suspension. Samples were resolved on 10 % gels and visualized with the PC1 antibody as described in the methods.
**Figure 30: Western Blot Analyses of SNAP-25 Following Differential Centrifugation in AtT-20 WT and PAM-1 Cells.** AtT-20 WT and PAM-1 cells were cultured for four days prior to the experiment, treated as described, and differential centrifugation was performed. Sample H was 1% of the total suspension, the P2 and P3 samples were 21% of total suspension, the P4 sample was 6% of the total suspension, and the C sample was 2% of total suspension. Samples were resolved on 10% gels and visualized with the SNAP-25 antibody (1:500) as described in the methods.
These results suggest that the plasma membrane is found mainly in the P2 and P3 pelleted samples.

Lastly, the clathrin coated pit marker, α-adaptin was used and detected at 112 kDa with the α-adaptin antibody (Figure 31). In the AtT-20 WT control, BCS, copper and BCS/copper cells, α-adaptin was observed in the H, and P2 and P3 pelleted samples. In the BCS, copper, and BCS/copper treated cells, α-adaptin was also detected in the cytosolic (C) sample. And perhaps due to the over-exposure of the BCS/copper blot, α-adaptin was observed in P4. In the At-T 20 PAM-1 cells, regardless of treatment, the localization pattern for α-adaptin was similar. In all four blots, α-adaptin was observed in the H, P2-P4 and C samples. Taken together, these results suggest that in AtT-20 WT cells, the plasma membrane was found in the P2 and P3 pelleted samples; however, in AtT-20 PAM-1 cells, the plasma membrane appeared to be in all samples.

Since these results demonstrated that the MNK protein was found in the P2 and P3 pellets and could be localized to areas corresponding to the TGN, secretory granules, and the plasma membrane, these two pellets were further analyzed. As described in the methods, the sucrose density gradients were designed so that P2 was enriched in mitochondria, TGN and some secretory granules. The P3 gradient was made so that it was enriched primarily in secretory granules with little TGN. From each of the fourteen layers, 30 µl of the P2 and P3 sucrose density gradients were analyzed by Western blot using the carboxyl-terminal MNK specific antibody (MNK) and organelle markers (PC1, SNAP-25 and α-adaptin) as described in the methods.

In the P2 sucrose density gradient fractions from the AtT-20 WT cells at steady-state, a MNK protein band (178kDa) was detected in fractions 8-14 with enrichment in fractions 10 and 11 as well as in 14 (Figure 32; top panel).
### Figure 31: Western Blot Analyses of α-Adaptin Following Differential Centrifugation in AtT-20 WT and PAM-1 Cells.

AtT-20 WT and PAM-1 cells were cultured for four days prior to the experiment and differential centrifugation was performed as described. The H sample was 1% of the total suspension, the P2 and P3 samples were 21% of the total suspension, the P4 sample was 6% of the total suspension, and the C sample was 2% of total suspension. Samples were resolved on 10 % gels and visualized with the α-adaptin antibody (1:500) as described in the methods.
Figure 32: The Localization of the MNK Protein and Organelle Markers from the P2 Sucrose Density Gradients in the AtT-20 WT Cells. AtT-20 WT cells were cultured for four days prior to the experiment and subcellular fractionation was performed as described. The P2 pellet was layered onto a P2 sucrose density gradient and centrifuged. Equal aliquots from each fraction (30 µl) were resolved on 12 % gels and visualized with the antibodies as shown.
The secretory granule protein, PC1, was detected using the PC1 antibody. Detection of the 80 kDa band of PC1 was observed in fraction 14 and the 60 kDa band was detected in fractions 10-14 (Fig. 32; 2\textsuperscript{nd} panel). SNAP-25, a plasma membrane protein marker, was detected as a 25 kDa band using the SNAP-25 antibody in fractions 5-14 (Fig. 32; 3\textsuperscript{rd} panel). Lastly, the clathrin coated pit marker, $\alpha$-adaptin, was detected as a 112 kDa band in fractions 8-14 (Fig. 32; 4\textsuperscript{th} panel).

In the P3 sucrose density gradient, the MNK protein was observed in fractions 6-14 with enrichment in fractions 12-14 (Figure 33; 1\textsuperscript{st} panel). The 80 kDa band of PC1 was detected in fraction 14 and the 60 kDa band was detected in fractions 9-14 (Fig. 33; 2\textsuperscript{nd} panel). SNAP-25 was detected in fractions 4-14 (Fig. 33; 3\textsuperscript{rd} panel) and $\alpha$-adaptin was detected in fractions 6-14 (Fig. 33; 4\textsuperscript{th} panel). Taken together, these results indicate that the MNK protein is found in fractions that overlap the organelle markers. Moreover, the MNK protein in the AtT-20 WT cells at steady-state is localized to secretory granules, the plasma membrane, and clathrin coated pits.

Since the MNK protein is a copper transporter and the localization of the MNK protein is copper dependent, the next step was to determine the localization of the MNK protein in the AtT-20 WT and PAM-1 cells when exposed to high or low levels of copper. P2 and P3 sucrose density fractions from the AtT-20 WT and PAM-1 cells treated with the copper chelator, BCS (50 $\mu$M), CuCl\textsubscript{2} (200 $\mu$M) or BCS/CuCl\textsubscript{2} were examined by Western blot analyses using the carboxyl-terminal MNK specific antibody (MNK) and various organelle markers (PC1, SNAP-25 and $\alpha$-adaptin) as described in the methods. In AtT-20 PAM-1 control and treated cells, the localization of the PAM-1 and PHM proteins were also examined.
Figure 33: The Localization of the MNK Protein and Organelle Markers from the P3 Sucrose Density Gradients in AtT-20 WT Cells. AtT-20 WT cells were cultured for four days prior to the experiment and subcellular fractionation was performed as described. The P3 pellet was layered onto a sucrose density gradient and centrifuged. Equal aliquots from each fraction (30 µl) were resolved on 12 % gels and analyzed by Western blot analyses using the antibodies shown.
In the P2 sucrose density gradient fractions from AtT-20 WT control cells, the MNK protein was observed in fractions 7-14 at 178 kDa (Figure 34; 1st left panel). In the BCS and BCS/copper treated cells, the MNK protein was observed in fractions 13 and 14 (Fig. 34; 2nd and 4th left panels). In the copper treated cells, the MNK protein was enriched in fractions 10-14 and a faint band was observed in the lighter fractions (Fig. 34; 3rd left panel). For the P3 sucrose density gradient from the AtT-20 WT cells, the MNK protein was detected in fractions 9-14, with enrichment in fractions 12-14 (Fig. 34; 1st right panel). In the BCS, copper and BCS/copper treated cells, the MNK protein was observed in the heavier fractions, 13 and 14 (Fig. 34; 2nd, 3rd, and 4th left panels). However, in the copper treated cells, the MNK protein also was observed in the lighter fractions of the gradient.

The localization of PC1, a secretory granule protein, in the P2 and P3 sucrose density gradients for AtT-20 WT cells appeared to be similar (Figure 35; left and right panels). For the control (Fig. 35; 1st left and right panels), BCS (Fig. 35; 2nd left and right panels), and copper (Fig. 35; 3rd left and right panels) treated cells from the P2 gradient, the 60 kDa PC1 band was observed in fractions 10-14 with a faint 60 kDa band observed in the lighter fractions, 1-2. The 80 kDa PC1 band was observed in the last fraction, 14. In the BCS/copper treated cells (Fig. 35; 4th left and right panels), the 60 kDa PC1 band was observed in fractions 12-14 as well as in fractions 1-3.

A SNAP-25 antibody was used to detect which fractions contained the plasma membrane protein, SNAP-25. In the P2 gradient from the control (Figure 36; 1st left panel), BCS (Fig. 36; 2nd left panel), and copper (Fig. 36; 3rd left panel) treated AtT-20 WT cells, SNAP-25 was observed in fractions 5-14 at 25 kDa. However, in the BCS/copper treated cells (Fig. 36; 4th left panel), SNAP-25 was detected in fractions 4-14. In the P3 gradient, there was a little more
Figure 34: The Localization of the MNK Protein from the P2 and P3 Sucrose Density Gradients in AtT-20 WT cells. AtT-20 WT cells were cultured for four days prior to treatment with BCS (50µM; 15 h), CuCl₂ (200µM; 5 h), or BCS/CuCl₂. Control cells were not treated. Subcellular fractionation was performed as described to obtain P2 (A) and P3 (B) pellets, which were layered onto sucrose density gradients. Equal aliquots from each fraction (30µl) were resolved on 12% gels. The MNK protein was detected using the MNK antibody (1:500) as described in the methods. The typical localization of the plasma membrane (PM), the trans-Golgi network (TGN) and secretory granules (SG) is delineated as shown.
Figure 35: The Localization of PC1 from the P2 and P3 Sucrose Density Gradients in AtT-20 WT cells. AtT-20 WT cells were cultured for four days prior to treatment with BCS (50 µM; 15 h), CuCl$_2$ (200 µM; 5 h), or BCS/CuCl$_2$, and subcellular fractionation was performed as described. P2 (A) and P3 (B) pellets were layered onto sucrose density gradients and centrifuged. Equal aliquots from each fraction (30 µl) were resolved on 12 % gels. PC1 was detected using the PC1 antibody (1:1000).
Figure 36: The Localization of SNAP-25 from the P2 and P3 Sucrose Density Gradients in AtT-20 WT Cells. AtT-20 WT cells were cultured for four days prior to treatment with BCS (50 µM; 15 h), CuCl₂ (200 µM; 5 h), or BCS/CuCl₂, and subcellular fractionation was performed. P2 (A) and P3 (B) pellets were layered onto sucrose density gradients and centrifuged. Equal aliquots from each fraction (30 µl) were resolved on either 10 % or 12 % gels. SNAP-25 was detected using the SNAP-25 antibody (1:500) as described in the methods.
variability in the fractions in which SNAP-25 was detected. In the control WT cells (Fig. 36; 1st right panel), SNAP-25 was detected in fractions 4-14. In the BCS (Fig. 36; 2nd right panel) and copper (Fig. 36; 3rd right panel) treated WT cells, SNAP-25 was found in fractions 5-14. Lastly, in the BCS/copper treated cells (Fig. 36; 4th right panel), SNAP-25 was observed in fractions 7-14 and 2.

α-Adaptin was used to detect clathrin coated pits in the endocytic and secretory pathways. In the P2 gradient from the control (Figure 37; 1st left panel) and BCS/copper (Fig. 37; 4th left panel) treated AtT-20 WT cells, α-adaptin was observed in fractions 8-14 at 112 kDa. Furthermore, in the BCS (Fig. 37; 2nd left panel) and copper (Fig. 37; 3rd left panel) treated WT cells, α-adaptin was observed in fractions 9-14. In the P3 gradients from AtT-20 WT cells, the blots visualized with the α-adaptin antibody produced some variability between the control and BCS WT cells versus the copper and BCS/copper treated cells. In the control (Fig. 37; 1st right panel) and BCS (Fig. 37; 2nd right panel) treated cells, α-adaptin was detected in fractions 8-14. However, in the copper (Fig. 37; 3rd right panel) and BCS/copper (Fig. 37; 4th right panel) treated cells, α-adaptin was observed in fractions 12-14.

To further evaluate the localization of the MNK protein at steady-state as well as in response to various copper levels, AtT-20 PAM-1 cells also were used. Subcellular fractionation and Western blot analyses of both the P2 and P3 sucrose density gradients were preformed as described in the methods using the same procedure as for the AtT-20 WT cells. These results were used to help determine if the MNK and PAM proteins are co-localized in the same subcellular organelles as well as to determine the localization of the MNK protein in response to copper levels in cells expressing high levels of a cuproenzyme.
Figure 37: The Localization of $\alpha$-Adaptin from the P2 and P3 Sucrose Density Gradients in AtT-20 WT Cells. AtT-20 WT cells were cultured for four days prior to treatment with BCS (50 $\mu$M; 15 h), CuCl$_2$ (200 $\mu$M; 5 h), or BCS/CuCl$_2$, and subcellular fractionation was performed. P2 (A) and P3 (B) pellets were layered onto sucrose density gradients and centrifuged. Equal aliquots from each fraction (30 $\mu$l) were resolved on 12 % gels. $\alpha$-Adaptin was detected using the $\alpha$-adaptin antibody (1:1000) as described in the methods.
Figure 38: The Localization of the MNK Protein and Organelle Markers from the P2 Sucrose Density Gradients in AtT-20 PAM-1 Cells. AtT-20 PAM-1 cells were cultured for four days prior to the experiment and subcellular fractionation was performed as described. The P2 pellet was layered onto a sucrose density gradient and centrifuged. Equal aliquots from each fraction 1 through 14 (30 µl) were loaded onto 12 % gels and analyzed by Western blot using the antibodies shown above.
In the P2 sucrose density gradient of AtT-20 PAM-1 cells at steady-state, the MNK protein (178 kDa) was detected in fractions 2-14 with enrichment in fractions 8-14 (Figure 38; 1st panel). Both the PAM-1 (120 kDa) and PHM (45 kDa) proteins detected using the PHM 1764 antibody were observed in fractions 5-14 (Fig. 38; 2nd panel).

The 80 kDa band of PC1 was detected in fractions 5-14, while the 60 kDa PC1 band was detected in fractions 1-14 (Fig. 38; 3rd panel). SNAP-25, the plasma membrane marker, was observed in fractions 4-14 (Fig. 38; 4th panel). P65, another vesicle marker, was localized to fractions 4-14 (Fig. 38; 5th panel). Lastly, α-adaptin, a clathrin coated pit marker, was seen in fractions 7-14 (Fig. 38; 6th panel).

For the AtT-20 PAM-1 cells at steady-state, the P3 sucrose density gradient also was examined by Western blot analyses. The MNK protein was detected in fractions 5-14, with enrichment in fractions 6-10 and 14 (Figure 39; 1st panel). Both the PAM-1 (120 kDa) and PHM (45 kDa) proteins were observed in fractions 7-14 (Fig. 39; 2nd panel). The 80 kDa band of PC1 was detected in fractions 5-14, while the 60 kDa PC1 band was detected in fractions 1–14 (Fig. 39; 3rd panel). SNAP-25 was observed in fractions 4-14 (Fig. 39; 4th panel), and P65 was detected in fractions 4-14 (Fig. 39; 5th panel). Finally, α-adaptin was seen in fractions 8-14 (Fig. 39; 6th panel). These results also suggest that in AtT-20 PAM-1 cells at steady-state that the MNK protein overlaps several organelles in the gradients. Moreover, both the PAM-1 and MNK proteins are found in the same fractions, suggesting the possible co-localization of the MNK protein and the cuproenzyme, PAM.

The trafficking and localization of the MNK protein in the AtT-20 PAM-1 cells in response to high or low levels of copper also was examined by subcellular fractionation and Western blot analyses as described in the methods. In the P2 and P3 sucrose density gradients
Figure 39: The Localization of the MNK Protein and Organelle Markers from the P3 Sucrose Density Gradients in AtT-20 PAM-1 Cells. AtT-20 PAM-1 cells were cultured for four days prior to the experiment and subcellular fractionation was performed as described. The P3 pellet was layered onto a sucrose density gradient and centrifuged. Equal aliquots from each fraction (30 µl) were resolved on 12 % gels and analyzed by Western blot using the antibodies shown.
**Figure 40: The Localization of the MNK Protein from the P2 and P3 Sucrose Density Gradients in AtT-20 PAM-1 Cells.** AtT-20 PAM-1 cells were cultured for four days prior to treatment with BCS (50 µM; 15 h), CuCl2 (200 µM; 5 h), or BCS/CuCl2. Control cells were not treated. Subcellular fractionation was performed as described to obtain P2 (A) and P3 (B) pellets, which were layered onto sucrose density gradients and centrifuged. Equal aliquots from each fraction (30 µl) were resolved on 12 % gels. Western blot analyses were performed using the MNK antibody (1:500). The typical localization of the plasma membrane (PM), the *trans*-Golgi network (TGN) and secretory granules (SG) is delineated as shown.
from the BCS treated AtT-20 PAM-1 cells, the MNK protein was localized in fractions 7-14 (Figure 40; 2nd left and right panel). Meanwhile, for the P2 copper and BCS/copper sucrose density gradients, the MNK protein was observed in fractions 9-14 (Fig. 40; 3rd and 4th left panels). In the P3 copper and BCS/copper sucrose density gradients, the MNK protein was observed in fractions 4-14 (Fig. 40; 3rd and 4th right panels).

In the P2 sucrose density gradient from the BCS treated PAM-1 cells, the PAM-1 (120 kDa) and PHM (45 kDa) proteins were detected in fractions 5-14, with enrichment in the lower fractions (Figure 41; 2nd left panel). In the copper (Fig. 41; 3rd left panel) and BCS/copper (Fig. 41; 4th left panel) treated cells, PAM-1 was detected in fractions 6-14, while PHM was detected in fractions 8-14. Interestingly, in the BCS treated PAM-1 cells, the intensity of the 45 kDa PHM band was increased when compared to the control, copper or BCS/copper treated P2 sucrose density gradients. In the P3 sucrose density gradient from the BCS, copper and BCS/copper treated cells (Fig. 41; 2nd, 3rd, 4th right panels), the PAM-1 protein was localized to fractions 8-14, while 45 kDa PHM was observed in fractions 10-14. Again, in the BCS treated cells, more PHM was detected.

For the P2 and P3 gradients from the BCS (Figure 42; 2nd right and left panels) and copper (Fig. 42; 3rd left and right panels) treated AtT-20 PAM-1 cells, the 60 kDa PC1 band was found throughout the gradients, whereas the 80 kDa PC1 band was found only in fractions 10-14. In the BCS/copper treated cells, the 60 kDa PC1 band was observed in fractions 10-14, and the 80 kDa PC1 band was observed in fractions 11-14 (P2) and 14 (P3). The localization of SNAP-25 in the P2 sucrose density gradient for the BCS treated PAM-1 cells was in fractions 4-14 (Figure 43; 2nd left panel). In the P3 sucrose density gradient for the BCS treated cells, SNAP-25 was observed in fractions 8-14 (Fig. 43; 2nd right panel). In the copper treated cells for the P2
and P3 sucrose density gradient, SNAP-25, was localized to fractions 3-14 (Fig. 43; 3\textsuperscript{rd} right and left panels), while in the BCS/copper treated cells for both gradients, SNAP-25 was observed throughout the gradients (Fig. 43; 4\textsuperscript{th} right and left panels). Finally, $\alpha$-adaptin in the P2 and P3 sucrose density gradients from the PAM-1 cells was observed in fractions 8-14 (Figure 44; 2\textsuperscript{nd} right and left panels). Meanwhile, in the copper and BCS/copper (treated cells for the P2 and P3 sucrose density gradients (Fig. 44; 3\textsuperscript{rd} and 4\textsuperscript{th} right and left panels), $\alpha$- adaptin was detected in fractions 7-14. Overall, these data indicate that the MNK protein overlaps many of the organelle markers and that is localized along with PAM in secretory granules.
Figure 41: The Localization of the PAM-1 and PHM Proteins from the P2 and P3 Sucrose Density Gradients in AtT-20 PAM-1 Cells. AtT-20 PAM-1 cells were cultured for four days prior to treatment with BCS (50 µM; 15 h), CuCl₂ (200 µM; 5 h), or BCS/CuCl₂. Subcellular fractionation was performed as described. The P2 (A) and P3 (B) pellets were layered onto sucrose density gradients and centrifuged. Equal aliquots from each fraction (30 µl) were resolved on 12 % gels. Western blot analyses were performed using the PHM antibody (1:1000) as described in the methods.
Figure 42: The Localization of PC1 from the P2 and P3 Sucrose Density Gradients in AtT-20 PAM-1 Cells. AtT-20 PAM-1 cells were cultured for four days prior to treatment with BCS (50 µM; 15 h), CuCl₂ (200 µM; 5 h), or BCS/CuCl₂ and subcellular fractionation was performed as described. The P2 (A) and P3 (B) pellets were layered onto sucrose density gradients and centrifuged. Equal aliquots from each fraction (30 µl) were resolved on 12 % gels. Western blot analyses were performed using the PC1 antibody (1:10000) as described in the methods.
Figure 43: The Localization of SNAP-25 from the P2 and P3 Sucrose Density Gradients in AtT-20 PAM-1 Cells. AtT-20 PAM-1 cells were cultured for four days prior to treatment with BCS (50 µM; 15 h), CuCl₂ (200 µM; 5 h), or BCS/CuCl₂, and subcellular fractionation was performed. The P2 (A) and P3 (B) pellets were layered onto sucrose density gradients and centrifuged. Equal aliquots from each fraction (30 µl) were resolved on 12 % gels. Western blots were performed using the SNAP-25 antibody (1:500) as described in the methods. The P2 sucrose density gradient Western blots are slightly overexposed.
Figure 44: The Localization of α-Adaptin from the P2 and P3 Sucrose Density Gradients in AtT-20 PAM-1 Cells. AtT-20 PAM-1 cells were cultured for four days prior to treatment with BCS (50 µM; 15 h), CuCl₂ (200 µM; 5 h), or BCS/CuCl₂, and subcellular fractionation was performed. The P2 (A) and P3 (B) pellets were layered onto sucrose density gradients and centrifuged. Equal aliquots from each fraction (30 µl) were resolved on 12 % gels. Western blot analyses were performed using the α-adaptin antibody (1:1000) as described in the methods.
Discussion

Previous studies focused on the localization and trafficking of the MNK protein in Madin-Darby canine kidney, HeLa, and CHO cells, as well as in fibroblasts from $Mo^{br}$ mice and human fibroblasts transfected with the MNK protein, have illustrated that the MNK protein is localized to the TGN at steady-state (Yamaguchi et al., 1996; Ambrosini and Mercer, 1999; Goodyer et al., 1999; Kim et al., 2003; Petris et al., 2002b; Greenough et al., 2004; Lane et al., 2004). Studies by Steveson and colleagues (2003) have shown in whole pituitary as well as in primary pituitary endocrine cells from $Mo^{br}$ mice, that the MNK protein is localized at steady-state to the TGN. Furthermore, it has been established that the MNK protein and the copper requiring enzyme, PAM, are endogenously expressed in the pituitary and that PAM function is altered in pituitary tissue expressing a non-functional MNK protein (Steveson et al., 2003). The present studies utilized the adrenocorticotropic tumor cell lines, AtT-20 WT and AtT-20 PAM-1, to examine the localization and trafficking of the MNK protein in the presence or absence of the PAM protein. This mouse pituitary tumor cell line was chosen because it contains endogenous levels of the MNK protein, it is easy to transfect, and more importantly it has a regulated secretory pathway (Milgram et al., 1992; Milgram et al., 1993). These studies were aimed at determining if the localization of the MNK protein is cell type specific, examining differences in the trafficking patterns of the MNK protein, and gaining a better understanding of the relationship between the localization of the MNK protein and the copper dependent enzyme, PAM, in pituitary cells. The results of these studies are summarized in Tables 3 and 4.

The first goal of this project was to establish the localization of the MNK protein in the AtT-20 WT and PAM-1 cells at steady-state as well as to determine whether the localization pattern was different in the two cell types. Immunofluorescence microscopy using a carboxyl-
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Table 3: A Comparison of the Localization of the MNK Protein in AtT-20 WT Cells by Immunofluorescence Microscopy and Subcellular Fractionation Followed by Western Blot Analyses.
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Table 4: A Comparison of the Localization of the MNK Protein in AtT-20 PAM-1 cells by Immunofluorescence Microscopy and Subcellular Fractionation Followed by Western Blot Analyses.
terminal specific MNK antibody showed that in both cell types, the MNK protein was detected mainly in the perinuclear region with some diffuse punctate staining observed throughout the cytoplasm. The perinuclear localization of the MNK protein was similar to the localization of TGN38, a TGN marker, as well as to that of PAM in the AtT-20 PAM-1 cells. Furthermore, the diffuse staining observed throughout the cytoplasm is similar to that observed with the CGA and PC1 antibodies, both secretory granule markers. The MNK protein was not localized primarily to clathrin coated pits or the plasma membrane as compared to the localization of the following proteins: α-adaptin (clathrin coated pits) and SNAP-25 (plasma membrane). In terms of the TGN localization, these results are in agreement with immunofluorescence microscopy studies in CHO cells (Dierik et al., 1997; Petris et al., 2002), HeLa cells (Yamaguchi et al., 1996), normal mouse fibroblasts and Moβr fibroblasts (La Fontaine et al., 1999), where the MNK protein is localized to the TGN at steady-state. However, the apparent localization of the MNK protein to secretory granules is a novel finding, which needs to be further explored. The similar localization of the MNK protein in the AtT-20 WT and PAM-1 cells suggests that having extra copies of the cuproenzyme PAM does not alter the localization of the MNK protein at steady-state. Although no significant MNK protein staining was observed in the tips of the processes in the AtT-20 cells, where secretory granules primarily are located, the possibility of the MNK protein being localized in secretory granules is highly likely and cannot be ruled out because AtT-20 cells do not contain significant numbers of secretory granules (El Meskini et al., 2001).

In an attempt to further refine the localization of the MNK protein in the AtT-20 WT and PAM-1 cells, subcellular fractionation by differential and sucrose density equilibrium centrifugation were performed to identify the subcellular compartments in which the MNK protein resides. Interestingly, the presence of the organelle markers, including PC1 (secretory
granules), SNAP-25 (plasma membrane), and α-adaptin (clathrin coated pits) in the P2 and P3 pellets suggests that the MNK protein also is present in other subcellular compartments in addition to its predominant localization in the TGN, thus confirming our immunostaining data.

Sucrose density gradient fractions of the P2 and P3 pellets from the AtT-20 WT and PAM-1 cells shows the presence of the MNK protein in both lighter and denser regions of the sucrose gradients. The MNK protein in the denser regions of the gradients co-distributes with the PAM-1, PHM and PC1 proteins, again indicating that in AtT-20 cells, the MNK protein is localized to secretory granules. Since the PAM-1 protein at steady-state also is localized to the perinuclear region overlapping the TGN marker as well as to secretory granules, the co-localization of the PAM and MNK proteins in the lighter region of the P2 and P3 gradients in the AtT-20 PAM-1 cells is indicative of the co-localization of the MNK and PAM-1 proteins in the TGN. These observations are confirmed by the immunofluorescence microscopy data in which a diffuse punctate staining was observed in the cytoplasm of the AtT-20 WT and PAM-1 cells, indicative of secretory granules. In contrast to the findings in the fibroblasts, these data clearly establish that in cells containing a regulated secretory pathway, the MNK protein is localized to secretory granules as well as the TGN.

Previous studies have shown that the MNK protein is present in the TGN in fibroblasts and CHO cells transfected with the MNK protein and cells from patients with Menkes disease at steady-state or in basal levels of copper. However, the MNK protein re-localizes from the TGN to the plasma membrane when cells are incubated with high extracellular copper levels; the MNK protein then traffics from the plasma membrane back to the TGN when copper levels are lowered (La Fontaine et al., 1998a; Voskoboinik et al., 1998; Suzuki and Gitlin, 1999; Pascale et al., 2003; Pase et al., 2004). Therefore, to determine if the MNK protein traffics in response to
high and low levels of copper in the AtT-20 WT and PAM-1 cells, the cells were incubated with BCS, copper or BCS/copper and subjected to immunofluorescence microscopy and subcellular fractionation analyses. In both cell types treated with the copper chelator, BCS, the MNK protein was observed in a the perinuclear region, indicative of the TGN, suggesting that when cells are incubated with lower extracellular copper levels, the MNK protein remains in the TGN. Interestingly, in the AtT-20 PAM-1 cells, the MNK protein appeared to be localized to a more tightly compact region of the TGN than in the WT cells. However, when the cells were incubated with high extracellular copper levels, the MNK protein was still located predominately in the perinuclear region; however, additional diffuse punctate staining throughout the cytoplasm also was observed. Although this increase in punctate staining indicates that some movement of MNK into vesicular structures occurred, the movement of the MNK protein to the plasma membrane in AtT-20 cells was much less than occurs in fibroblasts transfected with MNK and incubated in similar levels of extracellular copper (La Fontaine et al., 1998a).

To further characterize the trafficking of the MNK protein in both cell types, subcellular fractionation and Western blot analyses were performed following incubation of the AtT-20 cells with high and low extracellular copper levels. In the cells treated with BCS, the MNK protein was enriched in fractions 12-14, in the P2 and P3 gradients, whose densities correspond to the TGN and secretory granules. These data confirm the localization of the MNK protein in the TGN in lower extracellular copper levels as observed by immunostaining. In contrast, a wider distribution of the MNK protein was detected with increasing amounts in the lighter fractions of P2 and P3 sucrose gradients observed following incubation of the cells in high extracellular copper levels when compared to control and BCS treated cells. Since, in both cell types, the MNK protein in lighter fractions overlaps with the clathrin coated pit marker, α-adaptin, as well
as the plasma membrane marker, SNAP-25, the presence of the MNK protein in these fractions following copper treatment again indicates some movement of the MNK protein to the plasma membrane in response to copper as occurs in fibroblasts (La Fontaine et al., 1998). The presence of the MNK protein in these lighter fractions is in agreement with the more diffuse immunostaining observed in the AtT-20 cells following incubation with high extracellular levels of copper. Interestingly, after the BCS/copper treatment, the MNK protein also shows a somewhat wider distribution into lighter fractions when compared to BCS treated cells. These results indicate that treatment of both the WT and PAM-1 cells with high extracellular levels of copper in part reverts the effects of the copper chelator, BCS. However, the concentration of copper used for these studies is not sufficient to completely cause the MNK protein to traffic back to the plasma membrane as observed in fibroblasts transfected with the MNK protein (La Fontaine et al., 1998). In addition, no apparent differences are observed between trafficking of the MNK protein in AtT-20 WT and PAM-1 cells in response to various copper levels, it will be necessary to perform further studies to better analyze the effects, if any, of the presence of the cuproenzyme, PAM, on the trafficking of the MNK protein.

Within endocrine cells, there are two main secretory pathways, the constitutive and regulated, that transport newly synthesized proteins from the ER to the plasma membrane (Halban and Irminger, 1994; Traub and Kornfeld, 1997). In cells, such as fibroblasts, that only contain a constitutive pathway of secretion, proteins are sorted into constitutive secretory granules that are spontaneously exocytosed from the cell. In contrast, in cells such as AtT-20 cells, with both constitutive and regulated secretory pathways, newly synthesized proteins are either sorted to the constitutive pathway or are sorted, stored and secreted in response to an external signal in the regulated secretory pathway. Since the MNK protein is localized to the
TGN and secretory granules, a novel finding, in the endocrine AtT-20 cells, we hypothesize that the MNK protein contains specific routing determinants to account for its differential localization in these cells. Further studies will need to be developed to investigate the sorting and trafficking of the MNK protein in endocrine cells.
Chapter 2

MNK PROTEIN EXPRESSION DURING DEVELOPMENT IN BRAIN AND ENDOCRINE TISSUES

Mutations in ATP7A, the MNK gene, result in the X-linked copper deficiency disorder in humans known as Menkes disease. This disorder is characterized by an accumulation of copper in the intestines as well as the failure of copper to be distributed to other tissues, such as the brain and liver (Danks et al., 1972). Symptoms for this lethal disorder include neurodegeneration, connective tissue abnormalities, growth problems, mental retardation, loose skin, fragile hair, and hypopigmentation (Kaler, 1998; Mercer, 1998; Harrison and Dameron, 1999; Kodama and Murata, 1999). As previously detailed, patients diagnosed with Menkes disease as well as the Mo<sup>br</sup> mice, a mouse model for Menkes disease, have similar symptoms. Some of these symptoms are the direct result of cuproenzymes failing to receive the copper needed for them to function properly (Peña et al., 1999; Mercer 2001; Fatemi and Sarkar, 2002; Mercer and Llanos, 2003; Steveson et al., 2003; Rossi et al., 2004). The MNK protein has been shown to be at least partially responsible for the delivery of copper to the cuproenzyme, PAM. The failure of PAM to receive copper may lead to neurological and developmental deficits.

In humans and Mo<sup>br</sup> mice, copper injections given shortly after birth have been shown to increase survival rates and lessen some of the symptoms of the affected individuals (Mann et al., 1979; Yamano et al., 1985; Kodama et al., 2001; Kirodian et al., 2002; Kodama et al., 2005). MNK patients have low levels of circulating copper and copper concentrations (Koch et al., 1997; Mercer, 2001; Liu et al., 2005). Copper injections in the Mo<sup>br</sup> mice and in the brain of the macular mouse, another animal model for MNK disease, have been shown to cause a significant
increase in the activity of the cuproenzymes, lysyl oxidase and cytochrome c oxidase, as compared to non-treated mutant mice (Royce et al., 1982; Phillips et al., 1986; Kodama et al., 2001). Overall, the copper treated $Mo^{br}$ mice appeared to have less degeneration in the cerebellum and Purkinje cells than in the non-treated mutant mice (Nagara et al., 1981). These data suggest that copper treatments may increase the circulating copper in the body and the transport of copper across the blood brain barrier; therefore, alleviating some of the symptoms associated with MNK disease.

The majority of studies to date have focused on the expression of ATP7A and $Atp7a$ in tissues such as the brain, heart, lung, muscle, kidney, pancreas, and placenta from human Menkes patients and mouse models (Chelly et al., 1993; Vulpe et al., 1993; Levinson et al., 1994; Mercer et al., 1994; Murata et al., 1997; Paynter et al., 2003; Steveson et al., 2003). In addition, in situ hybridization and immunohistochemical studies have begun to examine MNK mRNA expression and localization during embryonic and postnatal development in WT and mutant $Mo^{br}$ mice (Iwase et al., 1996; Murata et al., 1997; El Meskini et al., 2005; Schlieff et al., 2006). In situ hybridization studies have demonstrated that in the $Mo^{br}$ mice, mutant MNK mRNA was expressed ubiquitously at different developmental stages (embryonic days 9.5, 11.5, 15.5 and 18.5), and in a variety of tissues (brain, heart, intestine, kidney, lung, and limbs) (Kuo et al., 1997; Murata, et al., 1997). Kuo and colleagues (1997) observed that MNK mRNA also was expressed in embryonic liver, but at much lower levels than in the adult liver from the $Mo^{br}$ mice. Furthermore, MNK mRNA expression levels in both the kidney and liver increased from postnatal day 2 to 17 in WT mice, while its expression levels in kidney and liver decreased during development in the mutant $Mo^{br}$ mice (Paynter et al., 1994).
Since Menkes disease is characterized by mental retardation, seizures, and poor eye movement as the result of severe neurodegeneration in the brain, studies have focused on the brain to further characterize ATP7A to begin to understand the reasons for the degeneration observed in patients with MNK disease (Iwase et al., 1996; Murata et al., 1997; Barnes et al., 2005). In situ hybridization of the brains of WT and macular mutant mice showed similarities in the expression of Atp7a at postnatal days 0, 4, 7, 10, and 13 (Iwase et al., 1996). This study further demonstrated that at postnatal day 13, in the macular mutant mice, the expression of MNK mRNA was lower in the hippocampal CA3 region and cerebellar Purkinje cells, and higher in the hippocampal CA1 region, the dentate gyrus, the cerebellar granular layer, and the olfactory bulb. Likewise, Nicui and colleagues (2006) observed a decrease in MNK mRNA in the cerebellum, the hippocampal CA1 and CA2 regions as well as the Purkinje cells in WT mice from postnatal day 4 to adult. In addition, these brain regions had begun to show signs of neurodegeneration, indicating that the altered expression of Atp7a was at least partially responsible for the symptoms presented in mice and patients with MNK disease.

To further examine the role of MNK during development, previous studies have examined MNK protein expression in the brain, heart, kidney, liver, lung, and small intestines in transgenic mice over-expressing the human MNK protein as well as in WT and Mo<sup>br</sup> mice (Grimes et al., 1997; Ke et al., 2005). Steveson and colleagues (2003) detected the expression of MNK protein in the adrenal gland, atrium, cortex, hypothalamus, and pituitary in postnatal day 10–12 WT and MNK mice. Moreover, in postnatal day 0, day 14, and 7 weeks (adult) WT mice, the MNK protein was expressed in the cerebellum, cortex, hippocampus, hypothalamus and olfactory bulb, with the highest expression at postnatal day 0. Further analyses determined that the MNK protein was expressed in the neocortex and cerebellum at postnatal days 0, 2, 4, 8,
11, 14 and 7 weeks, with a peak of MNK protein expression at postnatal day 4. Interestingly, while MNK mRNA expression in the cerebellar Purkinje cells and the hippocampal CA2 region decreased, MNK protein expression increased in these regions from postnatal day 4 to adult (Niciu et al., 2006).

Since the MNK protein has been shown to deliver copper to the cuproenzyme, PAM, and since we wish to explore the possibility that the MNK and PAM proteins are coordinately regulated during development, the expression of PAM expression also must be detailed. Northern blot analyses of PAM mRNA expression in rat adult tissues have shown that PAM is expressed in the adrenal glands, brainstem, cerebral cortex, heart, hippocampus, hypothalamus, kidney, lung, olfactory bulb, ovary, pituitary, retina, skeletal muscle, stomach, thalamus, and tongue (Braas et al., 1989; Stoffers, et al., 1991; Braas et al., 1992). In situ hybridization has determined that high expression levels of PAM mRNA exist in the telencephalon, diencephalon, thalamus, mesencephalon, pons, and medulla as well as the CA1, CA2, and CA3 regions of the hypothalamus, with little expression in the cerebellum (Schafer et al., 1992). As early as embryonic day 14, PAM mRNA is expressed as determined by Northern blot analyses, and continues to be expressed through postnatal day 45 in rat atria and ventricles. Interestingly, PAM mRNA expression levels that were the highest at embryonic day 14, were seen to decrease by postnatal day 3 and then increase again as the animal aged into an adult (Ouafik et al., 1989). Furthermore, immunohistochemistry studies were used to determine that PAM protein expression in mouse lung epithelia cells first occurred at embryonic day 16, which then increased during development through postnatal day 6 (Guembe et al., 1999).

PAM protein expression also has been reported in the olfactory epithelium, endothelial cells, bronchial cartilage, smooth muscle cells, brain ependymal cells and astrocytes, with
highest expression in adrenal medulla, atrial myocytes, the central nervous system, and pituitary (Eipper et al., 1989; Maltese and Eipper, 1992; Klein and Fricker, 1992; Oldham et al., 1992; Saldise et al., 1996; Zhang et al., 1997; Jaworsky et al., 1999). A few studies also have examined PAM protein expression in developing organs, such as in the rat heart and sheep pancreas (Maltese et al., 1989; Zhou and Thorn, 1990; Maltese and Eipper, 1992; Kapuscinski and Shilkes, 1995). For example, expression of the two catalytic domains of the PAM protein (PHM and PAL) in atrial cell cultures showed that PHM expression was the highest at postnatal day 14 and that PAL increased from postnatal day 5 to 14 (Maltese and Eipper, 1992).

The primary aim of the study presented in this chapter is for the first time to begin to investigate and understand if MNK is coordinately regulated during development along with a cuproenzyme, PAM, which depends on the activity of MNK for its function. Furthermore, we hope to begin to shed some light on the types of regulation MNK may be undergoing in brain and endocrine tissues during development. Thus, the expression of MNK mRNA and protein was examined at distinct developmental stages [postnatal day 3, day 30, and adult (+60 days)] in endocrine (adrenal glands, atria, and pituitary) and brain (cerebellum, cortex, hippocampus, hypothalamus, and medulla) tissues from rats. The expression of MNK in these tissues at these developmental time points then was compared to that of PAM expression in the same tissues and time points.
Methods

Animals and Care

Male and female post-natal day 3, day 30 and adult (+60 days) Sprague-Dawley rats (Harlan, Indianapolis, IN) were used for these studies (compliments of Dr. Lee Meserve, Biology Department, Bowling Green State University, Bowling Green, OH). The animals were raised in the Bowling Green State University, Animal Care Facility and given free access to food and water. All animals were maintained on a 12 h light/12 h dark cycle in a temperature monitored environment. All protocols were approved by the Bowling Green State University Animal Care and Use Committee (Bowling Green, OH).

Tissue and Cell Extract Preparation

Rats were killed by decapitation and the following tissues dissected: adrenal glands, atria, brain cortex, cerebellum, hippocampus, hypothalamus, brain medulla, and pituitary. The whole heart was removed from the postnatal day 3 animals, whereas only the atria were removed from the postnatal day 30 and adult animals. The whole pituitary gland was harvested from postnatal day 3 animals. In the day 30 and adult animals, the whole pituitary gland was harvested, placed in a dish containing Leibovitz L-15 medium (InVitrogen), the intermediate and anterior lobes of the pituitary separated under a dissecting microscope, and the anterior lobe of the pituitary used for this study. Tissues were frozen and stored at –80 °C before further analyses. Figure 45 diagrams the procedure for the preparation of tissue homogenates for performing Western blot analyses. The dissected tissues were weighed and homogenized in ice cold 10 mM TES Mannitol with 1% Triton (pH 7.4; TMT), containing the protease inhibitors PMSF and “X” using a Dounce homogenizer (Kontes, Vineland, NJ) kept on ice. Homogenates were frozen on
Extraction of tissues from postnatal day 3, day 30 and adult Sprague-Dawley Rats

Homogenization of dissected tissues in TMT with protease inhibitors

Samples centrifuged for 5 min at 13,000 rpm at 4 °C

Supernatant recovered and pellet discarded

Protein Assay

Fractionation of 30 µg of sample by SDS-PAGE gel electrophoresis

Western blot analyses using MNK and PHM antibodies

Figure 45: Flow Chart of Methods for the Analyses of the Developmental Expression of MNK and PAM Proteins in Sprague-Dawley Rats.
Dounce homogenizer (Kontes, Vineland, NJ) kept on ice. Homogenates were frozen on dry ice and thawed three times to break the cells, shear the membranes, and solubilize the proteins. Samples were centrifuged at 4 °C in a microfuge for 5 min at 13,000 rpm. Supernatants were frozen at –80 °C for further analyses.

**Protein Assays**

Protein assays were performed to determine the amount of protein in each tissue extract sample (mg/ml) using the bicinchonic acid protein reagent kit (BCA; Pierce Biotechnology, Rockford, IL). Aliquots of 10 µl and 20 µl of tissue extracts were analyzed in duplicate. Bovine serum albumin (BSA) standards of 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/ml (Pierce Biotechnology) were used to generate a standard curve. To make the reagent mix, one part of the BCA kit reagent, B, was added to 50 parts of BCA kit reagent, A. Samples were incubated for 30 min at 37 °C and the optical density determined in a spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 562 nm. The resulting readings of the BSA standards were used to produce the standard curve and protein concentrations of the samples were established based upon the standard curve using an Excel program developed by Dr. Richard Mains (University of Connecticut Health Center, Farmington, CT).

**Western Blot Analyses**

Western blot analyses were performed to determine the expression levels of the MNK and PAM proteins in the tissues. As previously described in Chapter 1 Methods, Western blot analyses were performed using 30 µg of each sample. The carboxyl-terminal specific MNK CT77 (1:1000) and PHM JH1764 (1:1000) polyclonal antibodies were used for these
experiments (compliments of Drs. B.A. Eipper and R.E. Mains). Detection was performed using the ECL method as previously described.

**Total RNA Isolation**

Total RNA was extracted from the tissue sets from postnatal day 3, day 30 and adult rats using the Trizol reagent (InVitrogen) according to the manufacturer’s specifications. Figure 46 diagrams the procedure for the isolation and reverse transcription–polymerase chain reaction (RT-PCR). Briefly, tissues were weighed, homogenized in Trizol (100 mg/ml) using a Potter-Elvehjem homogenizer, transferred to a new Eppendorf tube and incubated at room temperature for 10 min. Following incubation, the RNA was extracted by adding 200 µl of chloroform (Fisher Scientific, Fair Lawn, NJ) per 1 ml of homogenate, mixing vigorously, and incubating for 5-15 min at RT. Samples were centrifuged at 13,000 rpm for 15 min at 4 ºC to separate the colorless aqueous phase containing the RNA (top) from the pink phase of phenol/chloroform (bottom). The aqueous phase was transferred to a new tube and 0.5 ml of 2-propanol (Fisher Scientific) was added to precipitate the RNA. Samples were incubated 10-30 min at 4 ºC and centrifuged at 13,000 rpm for 10 min at 4 ºC. The supernatant was removed and the RNA pellet washed with 70% ethanol in RNase free water (ISC BioExpress, Kaysville, UT). Samples then were centrifuged at 10,000 rpm for 5 min. Alternatively, the samples were stored at -80 ºC overnight in 70% ethanol and the RNA was precipitated when needed. Following centrifugation, the ethanol was removed and the pellet was air dried for 10 min in a laminar air flow hood. The dried pellet was resuspended in 12 µl of RNase-free diethylpyrocarbonate water (ISC BioExpress, Kaysville, UT) and the RNA concentration was determined by measuring the optical density at 260 nm in a spectrophotometer (BioRad, Hercules, CA).
Figure 46: Flow Diagram of the Extraction of mRNA and RT-PCR
RT-PCR

Total RNA (1 µg) from each tissue set was used as a template for the RT-PCR reaction using a SuperScript One-Step RT-PCR kit (InVitrogen). MNK and β-actin sense and anti-sense primers (compliments of Dr. Ana Maria Oyarce) were used to determine the levels of MNK and β-actin mRNA, respectively. For the reaction cocktail, the following solutions were added to Eppendorf tubes: 2X Reaction Buffer (25 µl), total RNA (1 µg), 10 µM of the sense primer (1 µl), 10 µM of the anti-sense primer (1 µl), RT/Platinum Taq Polymerase (1.5 µl), and water to 50 µl. Tubes were placed in the thermocycler and the cycles were set as follows: Cycle 1 (2 min at 94 °C, and 45 min at 50 °C) was repeated 1X, Cycle 2 (1 min at 94 °C, 1 min at 55 °C, and 2 min at 68 °C) was repeated 36X, and Cycle 3 (1 min at 94 °C, and 20 min at 72 °C) was repeated 1X.

Following the RT-PCR, the samples were run on a 2.2 % Agarose/Tris-Acetate, ethylenediaminetetraacetic acid (EDTA) gel (10 mM Tris-HCl, pH 8.0, 0.1% glacial acetic acid, 0.2mM EDTA), containing 5 µl of Ethidium Bromide (10mg/ml; InVitrogen). The base pair DNA ladder was used for the standards (InVitrogen). The relative amounts of mRNA for MNK and β-actin were determined by scanning the agarose gel using a Typhoon Phosphoimager. A volume number of both the MNK and β-actin bands were obtained by Image Quant. The arbitrary number for the MNK band was divided by the arbitrary number of the β-actin, and this number was plotted on a graph (Molecular Dynamics program) in the Department of Pharmacology, Cardiovascular Biology and Metabolic Diseases, at the Medical University of Ohio, Toledo, OH under the supervision of Dr. Ana Maria Oyarce.
Results

Analyses of Tissue Extracts

To determine whether the expression of the MNK protein in atria, brain medulla, brain cortex, cerebellum, adrenal, pituitary, hypothalamus, and hippocampus is tissue-specific and coordinately regulated along with PAM protein expression during development, tissue extracts from post-natal day 3 (3 d), day 30 (30 d) and adult (A) Sprague-Dawley rats were analyzed by Western blot using the MNK and PHM antibodies. To better understand the forms and processed products of PAM, Figure 47 has been included. PAM-1 is comprised of two catalytic domains, PHM and PAL separated by a non-catalytic domain (Exon A), and followed by a transmembrane domain (TMD), and a carboxyl-terminal domain (CD). PAM-2 contains the two catalytic domains, PHM and PAL, the TMD, and CD, but lacks Exon A. Figure 47 also illustrates that the PHM antibody is able to detect PAM-1 and PAM-2 as well as the cleavage products, soluble PAM-2 (sPAM-2) and PHM.

In all the tissues (Figures 48 and 49), MNK was detected as a 178 kDa band with the MNK antibody. In the hippocampus, MNK expression decreased from postnatal day 3 to adult (Fig. 48A). In the brain medulla, MNK expression remained unchanged from postnatal day 3 to adult (Fig. 48B). Alternatively, in the brain cortex and cerebellum, MNK expression decreased from postnatal day 3 to adult (Fig. 48C and D). In terms of PAM expression in the hippocampus, PAM-1 (120 kDa), sPAM-2 (70 kDa) and PHM (45 kDa) were detected with the PHM antibody in postnatal day 30 and adult with little expression of these proteins observed at postnatal day 3 (Fig. 48A). In the hippocampus, there was more non-specific binding of the antibody, as noted by the extra bands (*). In the brain medulla, little PAM-1, PAM-2 and sPAM-2 were
Figure 47: PAM Proteins and their Cleavage Products.
Peptidylglycine α-amidating monooxygenase-1 (PAM-1) is composed of peptidylglycine α-hydroxylating monooxygenase (PHM), Exon A, peptidyl α-hydroxyglycine α-amidating lyase (PAL), a transmembrane domain (TMD) and a cystolic domain (CD); it is the largest form of PAM at 120 kDa. PAM-2 lacks the Exon A domain; thus, it is smaller in size at 105 kDa. The cleavage of PAM-1 yields PHM (45 kDa), and the cleavage of PAM-2 yields sPAM-2 (70 kDa), which lacks the TMD and CD. The PHM antibody detects PAM-1, PAM-2, sPAM-2, and PHM. (Modified from Ciccotosto et al., 2000).
Figure 48: MNK and PAM Protein Expression in Brain Tissues.
Hippocampus (A), brain medulla (B), brain cortex (C) and cerebellum (D) were removed and weighed from postnatal day 3 (3 d), day 30 (30 d) and adult (A) Sprague-Dawley rats. Aliquots of extracted tissue samples (30 µl) were fractionated by SDS-PAGE and Western blot analyses performed using the MNK and PHM antibodies. The asterisk denotes non-specific binding of the PHM antibody.
detected, although there was a faint 45 kDa band of PHM observed at postnatal day 30 (Fig. 48B). In both the brain cortex and cerebellum, a 45 kDa band of PHM was the only PAM product detected, which was expressed at postnatal day 30 and remained unchanged in the adult (Fig. 48C and D). Overall, these results suggest that expression of both the MNK and PAM proteins is tissue specific. Moreover, the expression of the MNK and PAM proteins are not coordinately regulated during development in these brain tissues.

MNK expression was first detected in the adrenal glands at postnatal day 3 (3 d) and the expression increased from postnatal day 30 (30 d) to adulthood (A) (Fig. 49A). In the pituitary, MNK protein expression also increased from postnatal day 3 to 30, but then decreased in the adult (Fig. 49B). MNK expression was detected in the rat brain hypothalamus at postnatal day 3 and day 30, and then decreased in the adult (Fig. 49B and C). In the whole heart from the postnatal day 3 rats, MNK protein expression was first detected with levels of expression appearing to remain constant into adulthood in the atria (Fig. 49D). When examining PAM expression in the adrenal glands, PHM (45 kDa) expression was not observed at postnatal day 3, but was observed in postnatal day 30 tissue extracts and remained unchanged in the adult (Fig. 49A). In contrast, in the pituitary, at postnatal day 30 and in the adult animals, a strong band at 70 kDa corresponding to sPAM-2 was detected (Fig. 48A and B). In the hypothalamus, PAM-1 (120 kDa), sPAM-2 (70 kDa), and PHM (45 kDa) were all detected in the postnatal day 30 animals and adults (Fig. 49C). However, non-specific bands also were detected with the PAM antibody in the day 30 and adult hypothalamic tissue extracts (Fig. 49C, asterisk). At postnatal day 3 in the whole heart, PAM-1 and sPAM-2, were expressed, while in the atria from the postnatal day 30 and adult tissues, a stronger expression of PAM-1, sPAM-2, and PHM were
Figure 49: MNK and PAM Protein Expression in Endocrine and Brain Tissues. Adrenals (A), pituitary (B), hypothalamus (C) and atria (D) were removed and weighed from postnatal day 3 (3 d), day 30 (30 d) and adult (A) Sprague-Dawley rats. Aliquots of extracted tissue samples (30 µl) were fractionated by SDS-PAGE and Western blot analyses performed using the MNK and PHM antibodies. The asterisk denotes non-specific binding of the PHM antibody.
Analyses of RT-PCR

RT-PCR was carried out to determine the expression of MNK mRNA during rat development in brain and endocrine tissues (see Fig. 46). A RT-PCR product of 390 base pairs was observed for MNK and a 430 base pair fragment was observed for β-actin (Figure 50). MNK and β-actin mRNA was expressed in the brain cortex, brain medulla, hypothalamus, hippocampus, and cerebellum at postnatal day 3, day 30 and adult (Fig. 50A). A preliminary quantification was obtained by normalizing the relative levels of MNK mRNA to the control, β-actin mRNA. As observed in Fig. 50B, MNK mRNA expression increased in both the brain cortex and hypothalamus from the postnatal day 3 to the adult, but expression decreased in the cerebellum from day postnatal 3 to the adult (Fig. 50). Meanwhile, MNK mRNA expression levels in the brain medulla and hippocampus appeared to be unchanged during rat development (Fig. 50B).

RT-PCR also was performed on endocrine tissues to determine the expression of MNK mRNA during rat development (Figure 51A). As shown, MNK and β-actin mRNA were expressed in the endocrine tissues (pituitary, adrenal glands and heart/atria) in postnatal day 3 and day 30, and in the adult animals (Fig. 51A). For a rough quantification, relative levels of MNK mRNA were normalized to the level of an internal control, β-actin mRNA (Fig. 51B). As shown in Figure 51B, MNK mRNA expression decreased in pituitary from the postnatal day 3 to the adult animals. MNK mRNA expression appeared to be unchanged in the adrenal glands in
Figure 50: MNK mRNA Expression in Brain Tissues. MNK mRNA and β-actin expression (A) and quantification (B) were performed in rat brain tissues. Tissues were dissected and weighed from postnatal day 3, day 30 and adult Sprague-Dawley rats. Total RNA was extracted and used as a template to measure mRNA expression using RT-PCR. A 390 base pair fragment was observed for MNK and a 430 base pair fragment was detected for β-actin. Levels of MNK mRNA were normalized using the internal control, β-actin. Postnatal day 3 (3 or 3d), postnatal 30 (30 or 30d), adult (A), cortex (Cort.), medulla (Med.), hypothalamus (Hypo.), hippocampus (Hippo.), and cerebellum (Cereb.).
Figure 51: MNK mRNA Expression in Endocrine Tissues. MNK and β-actin mRNA (A) and quantification (B) were performed in rat endocrine tissues. Tissues were dissected and weighed from postnatal day 3, day 30 and adult Sprague-Dawley rats. Total RNA was extracted and used as a template to measure mRNA using RT-PCR. A 390 base pair fragment was observed for MNK and a 430 base pair fragment was detected for β-actin. Levels of MNK mRNA expression were normalized using the internal control, β-actin. Postnatal day 3 (3 or 3d), postnatal day 30 (30 or 30d), and adult (A), pituitary (Pit), adrenal (Adr), heart (Hrt), and atr (Atria).
the postnatal day 3, day 30 and adult animals. In addition, MNK mRNA expression decreased from day 3 to day 30, and levels increased from day 30 to the adult in the heart/atria (Fig. 51B).
Discussion

Previous studies have examined either MNK protein and/or mRNA expression in a variety of tissues (brain, lung, kidney, heart, and spleen) from humans, WT mice, mottled blotchy mice and Mo<sup>br</sup> mice (Grimes et al., 1997; Murata et al., 1997; Reed and Boyd, 1997; Steveson et al., 2003; Ke et al., 2005; Liu et al., 2005). Recently, studies have started to focus on the developmental regulation of MNK in the olfactory system (El Meskini et al., 2005) and brain (Niciu et al., 2006). However, no studies have fully examined whether there is a coordinated regulation of MNK protein and mRNA with the cuproenzyme, PAM, to which it has been shown to deliver copper during development (Steveson et al., 2003). The current study examined the expression of MNK and PAM at three development ages (postnatal day 3, day 30, and adult) in Sprague-Dawley Harlan rats.

In the first experiments (Figs. 48 and 49), in the postnatal day 3, day 30 and adult rat adrenal glands, heart/atria, brain cortex, cerebellum, hippocampus, hypothalamus, brain medulla, and pituitary, MNK was detected by the carboxyl-terminal MNK antibody as a 178 kDa band. Data from the Western blot analyses suggest that changes occur in MNK protein expression during development; however, the molecular mass of MNK remained unaltered. MNK protein expression in all the tissues listed above demonstrates that MNK protein expression is not tissue specific. In the second set of experiments (Figs. 50 and 51), we established that we could amplify a RT-PCR MNK product using MNK primers to produce a MNK sized product of 390 base pairs uniformly throughout all of the tissues. As an internal control, β-actin was used, amplified using β-actin primers, and a β-actin sized product of 430 base pairs was expressed in all tissues as expected for a housekeeping gene. In general, these RT-PCR analyses illustrate that MNK mRNA is differentially expressed during development in all the tissues examined.
Overall, as shown in Table 5, our data suggest that there is little parallel between the expression of the MNK protein and mRNA during development. In the adrenal glands, MNK protein expression increased through the three age points, whereas MNK mRNA expression appeared to remain unchanged from day 3 to adult. Thus, since MNK mRNA expression remained constant, while MNK protein expression changed, MNK expression could be translationally regulated in the adrenal glands. In the pituitary, both the MNK protein and mRNA expression decreased in the pituitary from the postnatal day 3 to adult animals. These data suggest that MNK expression is likely to be transcriptionally regulated in the pituitary. Finally, in the heart/atria, the other endocrine tissue we examined, MNK protein expression remained unchanged, while MNK mRNA expression decreased from postnatal day 3 to day 30 and then increased in the adult animals. This pattern of expression of MNK in the heart/atria might best be explained by regulation at the level of the message. Further analyses will be needed to confirm these patterns of expression, which vary greatly in the endocrine tissues we examined.

We also examined expression of MNK protein and mRNA in a variety of brain tissues (cerebellum, cortex, medulla, hippocampus, and hypothalamus; Table 5). In the hippocampus and cerebellum, MNK protein and mRNA expression decreases from postnatal day 3 to the adult animals, suggesting that MNK expression is transcriptionally regulated in these tissues as in the pituitary. In the hypothalamus, MNK protein and mRNA expression remained unchanged from postnatal day 3 to day 30 animals, indicating a lack of developmental regulation. However, in the adult hypothalamus, MNK protein expression appeared to decrease, while the message increased. Further analyses will be needed to confirm these patterns of expression. In both the brain cortex and medulla, MNK protein expression is unchanged; however, in the cortex, MNK
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Western Blot Analyses of MNK Protein</th>
<th>RT-PCR &amp; Quantification of MNK mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal Glands</td>
<td>Increased from 3 to A</td>
<td>Unchanged from 3 to A</td>
</tr>
<tr>
<td>Pituitary</td>
<td>Decreased from 3 to A</td>
<td>Decreased from 3 to A</td>
</tr>
<tr>
<td>Atria</td>
<td>Unchanged from 3 to A</td>
<td>Decreased from 3 to 30 and Increased in A</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>Unchanged from 3 to 30 and Decreased in A</td>
<td>Unchanged from 3 to 30 and Increased in A</td>
</tr>
<tr>
<td>Brain Cortex</td>
<td>Unchanged from 3 to A</td>
<td>Increased from 3 to A</td>
</tr>
<tr>
<td>Brain Medulla</td>
<td>Unchanged from 3 to A</td>
<td>Unchanged from 3 to A</td>
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<tr>
<td>Cerebellum</td>
<td>Decreased from 3 to A</td>
<td>Decreased from 3 to A</td>
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<tr>
<td>Hippocampus</td>
<td>Decreased from 3 to A</td>
<td>Unchanged from 3 to A</td>
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</tbody>
</table>

Table 5: Comparison of MNK Protein and MNK mRNA Expression. Postnatal day 3 (3), day 30 (30) and adult (A).
mRNA expression increased, while in the medulla, MNK mRNA expression remained unchanged. These data suggest that MNK expression is not developmentally regulated in the brain medulla. However, since changes in protein expression in the present data for the brain cortex are not clear, further experiments will be needed to assess the potential developmental regulation of MNK in these tissues.

These results along with experiments from the literature suggest that MNK is developmentally regulated in endocrine and brain tissues. Interestingly, Niciu and colleagues (2006) observed a decrease in MNK protein levels in brain cortex, hippocampus, olfactory bulb, cerebellum, and hypothalamus from postnatal day 0 to postnatal day 14 to 7 weeks (adult). Furthermore, their study demonstrated that MNK protein is the most highly expressed in the hippocampus and cerebellum. In addition, Steveson and colleagues (2003) demonstrated that at postnatal day 10-12, MNK protein is the most highly expressed in the pituitary and adrenal glands, with lower expression in the brain cortex, hypothalamus and hippocampus. While the general trends of our data agree with these studies, further quantitative analyses of our data are needed to corroborate these studies.

MNK mRNA expression patterns during development in different tissues also have been examined previously (Steveson et al., 2003; Paynter et al., 1994). Paynter and colleagues (1994) observed high levels of MNK mRNA expression in the placenta, lung, heart, brain and kidney in adult male mice. This study also demonstrated that MNK mRNA expression levels in kidney and liver tissues from mice decreased from day 17 to day 19, while little expression was observed at day 2. Steveson and colleagues (2003) demonstrated that in adult male mice, MNK mRNA is expressed in pituitary, hypothalamus, adrenal glands, heart, brain cortex, cerebellum, olfactory bulb, spleen, and liver, with the highest expression in the pituitary. The data collected
from the present study support these results in that MNK is indeed developmentally regulated and that expression depends on tissue type.

Of further interest, is that Steveson and colleagues (2003) demonstrated in WT and Mo<sup>br</sup> mice tissues expressing high MNK mRNA levels, that they also had high PAM expression levels. Moreover, Mo<sup>br</sup> mice, which lack a functional MNK protein, have altered PAM function resulting in a decrease of α-amidated peptides. In addition, studies have examined the expression of the PAM protein in the pituitary and atrium (Faivre-Bauman <i>et al.</i>, 1988; Ciccotosto <i>et al.</i>, 2000), pancreas (Ouafik <i>et al.</i>, 1987), and mRNA expression levels in the pituitary, heart, adrenal glands, lung, liver, spleen and kidney (Braas, <i>et al.</i>, 1989). Although, developmental studies have examined PAM expression in individual tissues such as the heart (Ouafik <i>et al.</i>, 1989; Maltese and Eipper, 1992), uterine smooth muscle (Zhang <i>et al.</i>, 1997), and lung (Guembe <i>et al.</i>, 1999), and since the symptoms of Menkes disease are partially due to inactive enzymes that need copper as a cofactor to be functional (Mercer <i>et al.</i>, 2001), it would be important to understand the developmental regulation of the cuproenzyme, PAM and its relationship to MNK.

To examine PAM protein expression levels in the heart/atria, adrenal glands, pituitary and brain tissues from rats, Western blot analyses were performed using the PHM antibody. Table 6 compares the expression of the MNK protein to that of the PAM proteins in the brain and endocrine tissues. In all the tissues, an increase in PAM expression and the production of cleavage products was observed with age. In the adrenal glands, there was an increase in sPAM-2 (70 kDa) and PHM (45 kDa). In the hypothalamus and hippocampus as well as in the heart/atria, from day 3 to adult, there was an increase in PAM-1 and PHM expression, while in the brain cortex and cerebellum, an increase in PHM was observed during development.
### Table 6: Comparison of MNK and PAM Protein Expression.
Postnatal day 3 (3), day 30 (30) and adult (A)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Western Blot Analyses of MNK Protein</th>
<th>Western Blot Analyses of PAM Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal Gland</td>
<td>Increased from 3 to A</td>
<td>sPAM-2 and PHM Increased from 3 to A</td>
</tr>
<tr>
<td>Pituitary</td>
<td>Decreased from 3 to A</td>
<td>sPAM-2 and PHM Increased from 3 to A</td>
</tr>
<tr>
<td>Atria</td>
<td>Unchanged from 3 to A</td>
<td>PAM-1, sPAM-2 and PHM Increased from 3 to A</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>Unchanged from 3 to 30 and Decreased in A</td>
<td>PAM-1 and PHM Increased from 3 to A</td>
</tr>
<tr>
<td>Brain Cortex</td>
<td>Unchanged from 3 to A</td>
<td>PHM Increased from 3 to A</td>
</tr>
<tr>
<td>Brain Medulla</td>
<td>Unchanged from 3 to A</td>
<td>Unchanged from 3 to A</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>Decreased from 3 to A</td>
<td>PHM at 30</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>Decreased from 3 to A</td>
<td>PAM-1 and PHM Increased from 3 to A</td>
</tr>
</tbody>
</table>
Notably, when PAM protein expression is compared to that of MNK, no significant coordinated regulation was observed in most of the tissues examined except in the adrenal glands, where an increase in both MNK and PHM expression was observed and in the hippocampus where a decrease in MNK and PHM expression was observed. Surprisingly, in the pituitary, MNK protein levels decreased, while sPAM-2 levels increased with the animal’s age. Overall, these results suggest that there is little similarity in the expression of MNK and PAM proteins between the brain and endocrine tissues.

The enzyme activity of PAM might be influenced by the levels of its expression as well as the forms of PAM expressed in different tissues. Braas and colleagues (1989) demonstrated that different tissues have different levels of soluble and membrane associated PAM activity, noting that membrane associated PAM activity in the anterior lobe of the pituitary was higher than in the cerebellum. More importantly, PAM activity and protein levels directly regulate the levels of amidated peptides, while CRH, TRH, GnRH and POMC play important roles in development. Interestingly, Ouafik and colleagues (1987) demonstrated that both PAM activity and TRH levels in the pancreas were the highest after birth and declined into adulthood in rats. Therefore, the possibility that PAM could be co-regulated with other proteins such as the MNK protein exists and needs to be investigated further.

The gain a better understanding of the regulation of MNK, the current study could be expanded. Using Western blot analyses, alternative antibodies, such as PC1, which is important in the post-translational processing of neuropeptides into mature forms, or carboxypeptidase (CPE), which also is involved in the biosynthesis of neuropeptides, could be used to determine if a coordinated regulation of MNK and processing enzymes occurs (Zheng et al., 1994). For the RT-PCR experiments, sense and anti-sense primers for CPE, PC1, and PHM also could be used.
to again determine if there is a coordinated transcriptional regulation of these proteins. The number of cycles could be adjusted to maximize the PCR reaction and prevent saturation, resulting in a more accurate measurement of mRNA levels. Finally, \textit{in situ} hybridization and immunocytochemical studies could be used to analyze the distribution of MNK and PAM in different tissues.

In summary, MNK was expressed differentially in endocrine and brain tissues at three developmental time points, with little similarity observed between the regulation of PAM and MNK expression in these tissues. Therefore, it is necessary to develop further studies to gain a better understanding of the regulation of MNK during development in endocrine and brain tissues. The ultimate goal of these studies would be to determine the best age to administer copper to patients with MNK disease, in hopes of alleviating some of the debilitating symptoms of this disease.
Chapter 3

LOCALIZATON AND EXPRESSION OF MNK IN STIMULATED ENDOCRINE CELLS

The hypothalamic-pituitary-adrenal axis (HPA axis) is essential for maintaining homeostasis and handling stress cues in animals. The hypothalamus is situated under the thalamus and located in the middle of the base of the brain, forming part of the ventricular area of the third ventricle. Several body organs, including the adrenal glands and the pituitary, are coordinated by the hypothalamus. The hypothalamus processes cues such as noise, fear and anxiety, leading to the regulation and release of hypothalamic peptides and hormones, which in turn regulate the pituitary. Beneath the hypothalamus, the pituitary gland lies in the sphenoid sinus cavity at the base of the skull in a region called the sella turcica. The posterior pituitary is comprised of neurons that extend from the hypothalamus, whereas the anterior pituitary is comprised of endocrine cells that secrete hormones into the blood. Pituitary hormones control body functions, including metabolism, growth, development, and lactation. Each hormone in the pituitary is released from a specific cell type in response to a specific hypothalamic-releasing hormone. Furthermore, each hormone secreted acts upon a target organ (Fig. 52) (Thorner et al., 1998; Aron et al., 2001).

As shown in Figure 52, the hypothalamic releasing hormones include CRH, GHRH, prolactin-releasing hormone (PRLH), TRH, and GnRH. In response to each hypothalamic-releasing hormone, each pituitary cell type secretes a corresponding hormone (Thorner et al., 1998; Aron et al., 2001). The anterior pituitary is composed of five endocrine cell types: corticotropes, somatotropes, lactotropes, gonadotropes and thyrotropes. Each cell type in turn
Figure 52: Hypothalamic-Releasing Hormones, Pituitary Cell Types and Secreted Hormones. The hypothalamus secretes releasing hormones that act on the pituitary to regulate the release of a corresponding hormone. The hypophysiotropic hormones include: corticotropin-releasing hormone (CRH), growth hormone-releasing hormone (GHRH), prolactin-releasing hormone (PRLH), thyrotropin-releasing hormone (TRH), and gonadotropin-releasing hormone (GnRH). The anterior pituitary is composed of different endocrine cells, including corticotropes, somatotropes, lactotropes, thyrotropes, gonadotropes, and folliculartropes, that secrete different hormones. The products secreted from anterior pituitary include: adrenocorticotropic hormone (ACTH), growth hormone (GH), prolactin (PRL), thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) (Thorner et al., 1998 ).
secretes a different hormone; corticotropes secrete adrenocorticotropic hormone (ACTH), lactotropes secrete prolactin (PRL), somatotropes secrete growth hormone (GH), gonadotropes secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH), and thyrotropes secrete thyroid stimulating hormone (TSH) (Thorner et al., 1998; Aron et al., 2001).

Interestingly, CRH and TRH are amidated by the cuproenzyme, PAM (Eipper et al., 1992; Giraud et al., 1992). Furthermore, El Meskini and colleagues (2000) demonstrated that several of the hypothalamic-releasing hormones (GnRH, GHRH, CRH and TRH) influence the activity of PHM, the secretion of PAM, and the processing of PAM, in the individual pituitary cell types. Furthermore, it has been demonstrated that estrogens can regulate PAM mRNA levels in the rat anterior pituitary (El Meskini et al., 1997). Similarly in rat anterior pituitary, thyroid hormones influence the levels of PAM activity as well as the expression of PAM (Ouafik et al., 1990). Lastly, TRH has been shown to alter PAM activity (Ouafik et al., 1987). Therefore, these studies indicate that the hypothalamic releasing-hormones as well as hormones secreted from the pituitary can regulate the activity of PAM and its expression.

Since the pituitary is under the control of the hypothalamus and the MNK and PAM proteins are endogenously expressed in the pituitary, then perhaps the hypothalamic-releasing hormones, such as CRH released from the hypothalamus, could influence the expression of both MNK and PAM. To examine the regulation and exocytosis of secretory pathway and granular-associated proteins, a pituitary adrenocorticotrope tumor cell line, AtT-20, in which MNK is endogenously expressed has been used for these studies. AtT-20 cells have the characteristics of corticotropes, and both endocrine and AtT-20 cells have a regulative secretory pathway, which can be stimulated by specific signals, such as CRH, to secrete ACTH from secretory granules. Furthermore, releasing-hormones, such as CRH and agents termed secretagogues, can influence
the synthesis, processing and secretion of PAM in AtT-20 cells (Thiele and Eipper, 1990; Giraud et al., 1992; Aoki et al., 1997; Ciccotosto et al., 1999).

To further examine exocytosis from different populations of secretory granules in the regulated secretory pathway of endocrine cells, two general secretagogues, barium chloride (BaCl₂) and phorbol myristate acetate (PMA), also were used for these studies. BaCl₂ has been shown to stimulate the secretion of PAM proteins from mature secretory granules, whereas PMA stimulates the secretion of PAM proteins from both immature and mature secretory granules in both AtT-20 and pituitary cells. Furthermore, these secretagogues have been shown to increase the activity of PHM (Ciccotosto et al., 1999; El Meskini et al., 2000). In general, studies have not been done to elucidate whether the same regulators of PAM protein expression and secretion, such as the hypothalamic-releasing hormones, BaCl₂ or PMA, may regulate the expression of MNK.

For these studies, we used AtT-20 WT and AtT-20 cells transfected with a PAM-1 construct. Since AtT-20 WT cells have a regulated secretory pathway and the expression, processing and exocytosis of PAM, a secretory granule protein, has been well documented, these cells were chosen for the secretagogue studies (Milgram et al., 1992; Milgram et al., 1993; Ciccotosto et al., 1999; Ferraro et al., 2005). Moreover, since the activity of the PAM protein is at least partially dependent upon its acquisition of copper from MNK and since previous studies have demonstrated that PAM can be regulated in response to secretagogues, we proposed that the expression and localization of the MNK protein also could be affected by secretagogues as detailed above in the AtT-20 cells. Furthermore, we proposed that differences in MNK expression and localization could occur between the stimulated AtT-20 WT and PAM-1 cells.
**Methods**

**Cells**

AtT-20 WT and AtT-20 PAM-1 cells were used for these experiments. Cells were maintained in growth medium and passed weekly as previously described in Chapter 1 Methods.

**Immunofluorescence Microscopy**

AtT-20 WT or PAM-1 cells were plated onto 3-four well slides coated with poly-L-lysine (Sigma) four days prior to treatment and fixation. On the fourth day, cells were washed three times for 0.5 h each with 500 µl of DMEM/F-12/CO₂ supplemented with 10% v/v BSA, 10% v/v Nu-Serum IV and 5 ml Penicillin-Streptomycin followed by incubation in a CO₂ incubator at 37 °C. One slide served as a control and received only medium for 0.5 h. Meanwhile, a second slide was incubated with medium containing 1 µM phorbol 12-myristate 13-acetate (PMA; Sigma), while a third slide was incubated with medium containing 1 mM barium chloride (BaCl₂; Sigma) during the same time period. After the incubation period, cells were rinsed once with medium and fixed according to the immunostaining protocol outlined in Chapter 1 Methods. Cells then were incubated with carboxyl-terminal specific MNK CT77 (1:500), PHM JH1764 (1:500) or PC1 JH888 (1:500) antibodies diluted in a 2 mg/ml BSA solution in PBS. Finally, the cells were incubated with GAR-FITC (1:1000) and examined as described in Chapter 1 Methods.

**Stimulation Experiments**

AtT-20 WT and PAM-1 cells were plated on a 12-well plate for four days prior to the start of the experiment (Fig. 53A and 54A). On the fourth day, cells were incubated in 500 µl of
**A.**

<table>
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**B.**

<table>
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<th>2&lt;sup&gt;nd&lt;/sup&gt; Basal</th>
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<th>Extract Cells</th>
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**Figure 53: Strategy for Examining the Expression of the MNK Protein in Response to PMA and BaCl₂ in AtT-20 WT and PAM-1 Cells.** A. AtT-20 WT and PAM-1 cells were grown on a 12-well plate for four days prior to the start of the experiment. Four wells served as controls and received only DMEM/F-12/CO₂/BSA medium during the stimulation period. The other eight wells received either PMA (1 µM) or BaCl₂ (1 mM) for 30 or 120 min in the above medium. B. Before the cells in (A) were treated with the secretagogues, they were incubated with 500 µl of DMEM/F-12/CO₂ for 4-6 h (Incub). Cells then were washed three times for 0.5 h each with DMEM/F-12/CO₂/BSA (W1, W2 and W3) and incubated at 37 °C. After washing, the medium was collected to determine if the secretagogues were functioning properly after two -1 h periods of basal secretion (1<sup>st</sup> and 2<sup>nd</sup> Basal), followed by either a 30 or 120 min period of stimulation using the secretagogues described in (A) or medium, before collecting the medium (thin arrows). Finally, the cells were extracted.
serum-free DMEM/F-12/CO₂ for 4-6 h to achieve equilibrium. Cells then were washed three times for 0.5 h with the 500 µl of the serum-free DMEM/F-12/CO₂ supplemented with 0.1 mg/ml BSA followed by incubation in a CO₂ incubator at 37 °C.

For the first basal secretion, 500 µl of DMEM/F-12/CO₂/BSA was added to the cells and they were incubated in a CO₂ incubator at 37 °C for 1 h. The medium then was collected and 2.5 µl of 40 mg/ml PMSF was added before the samples were centrifuged for 5 min at 10,000 x g in a microfuge (4 °C). The medium were transferred to a new tube containing 2.5 µl of the inhibitor X mix, the samples frozen on dry ice and stored at –80 °C for future use (Fig. 53A; 1st Basal). The second basal secretion medium was collected the same way (Fig. 53B; 2nd Basal). After the two basal periods, the secretagogues PMA, cyclic-adenine monophosphate (cAMP; Sigma), CRH (Sigma) and BaCl₂ were diluted in DMEM/F-12/CO₂/BSA to the following final concentrations: 1 µM PMA, 1 µM cAMP, 1 µM CRH and 1 mM BaCl₂. For the stimulation period, control wells received only medium while the stimulation wells received 500 µl of each solution, respectively. Cells were incubated for 0.5 (Fig. 53B), 1 (Fig. 54B) or 2 h (Fig. 53B) in a CO₂ incubator at 37 °C. The medium was collected from the wells and protease inhibitors added before centrifugation as described previously (Fig. 53B). Samples were frozen on dry ice for future use. Lastly, cells were extracted in 300 µl of TMT containing 3 µl of PMSF and 3 µl of the inhibitor X mix and frozen on dry ice (Fig. 53B and 54B). Cell extracts were frozen and thawed three times to shear membranes and solubilize the proteins, and centrifuged for 5 min in a cold centrifuge (4 °C) at 10,000 rpm (El Meskini et al., 2000). Samples were frozen at –80 °C before further analyses.
Figure 54: Strategy for Examining the Expression of the MNK Protein in Response to Various Secretagogues in AtT-20 WT and PAM-1 Cells. A. Cells were grown on a 12-well plate for four days prior to the start of the experiment. The top four wells served as the controls for each of the secretagogues and received serum-free DMEM/F-12/CO₂/BSA medium during the stimulation period. Four treatment wells received one of the following secretagogues: PMA (1 µM), BaCl₂ (1 mM), cAMP (1 µM), or CRH (1 µM). B. Before the cells in (A) were treated with the secretagogues, they were incubated with 500 µl of DMEM/F-12/CO₂ for 4-6 h (Incub). Cells then were washed three times for 0.5 h each with DMEM/F-12/CO₂/BSA (W1, W2 and W3) and incubated at 37 °C. After washing, the medium was discarded, followed by a 1 h period of stimulation using the secretagogues or medium before extracting the cells in TMT with protease inhibitors.
**Protein Assay**

Protein assays were performed to determine the amount of protein in each cell extract sample using the Pierce BCA kit, following the manufacture’s protocol as described in Chapter 2 Methods.

**Western Blot Analyses**

To analyze the expression or secretion of MNK, PAM, PC1 and tubulin from the medium and cell extract samples, Western blot analyses were performed as described in Chapter 1 Methods. Medium samples were concentrated using Microcon centrifugal filter devices (Millipore, Fisher). Aliquots of 100 µl of medium were placed in a Microcon sample reservoir in an Eppendorf tube and centrifuged in a microfuge (4 °C) for 30 min at 10,000 rpm. The reservoirs then were inverted into a new Eppendorf tubes, and re-centrifuged in a cold microfuge (4 °C) for 5 min. The samples were collected and stored for later use. The concentrated media samples as well as 30 µg of the cell extracts were analyzed by Western blot as described in the Chapter 1 Methods using the following antibodies: MNK CT77 (1:1000), PHM JH1764 (1:1000), PC 1 JH888 (1:1000), and tubulin (1:500; Sigma).
Results

Analyses of Immunofluorescence Microscopy

To determine if the localization of the MNK protein is altered in the AtT-20 cells in response to secretagogues that promote the exocytosis of secretory granules, the cells were treated with BaCl$_2$, which causes the release of mature secretory granules, and phorbol ester (PMA), which causes the release of both immature and mature secretory granules, for 1 h (El Meskini et al., 2001). Immunofluorescence microscopy was performed using the MNK antibody, and the PC1 antibody was used to mark the presence of secretory granules. In the AtT-20 WT control cells, the MNK protein was localized mainly to the perinuclear region (Fig. 55A; wide arrow). Meanwhile, in the PMA simulated AtT-20 WT cells, the MNK protein localized to both the perinuclear region as well to distinct punctate vesicles along the boundaries of the cell (Fig. 55C; wide arrow and double thin arrows). Interestingly, in the WT cells treated with BaCl$_2$, the MNK protein was again found in the perinuclear region as for the control cells (Fig. 55E; wide arrow). To demonstrate that the secretagogues stimulated exocytosis of the secretory granules, the secretory granule marker PC1 was used. In the control, PMA and BaCl$_2$ treated cells, the PC1 protein was localized in the perinuclear region as well as in distinct punctate vesicles in the processes and tips of the cells (Fig. 55B, D and F; arrowheads).

To determine if the localization of the MNK protein also could be altered in the AtT-20 cells transfected with PAM-1, stimulation experiments using PMA and BaCl$_2$ followed by immunofluorescence microscopy were performed. In the control, PMA and BaCl$_2$ treated cells, the MNK protein detected by the MNK antibody was localized to the perinuclear region (Fig. 55A, C, and E; wide arrows). To demonstrate the localization of secretory granules, the control, PMA and BaCl$_2$ treated cells were immunostained with the PHM antibody. In all the cells, the
Figure 55: Stimulation of AtT-20 WT Cells with PMA and BaCl₂.
Control (A and B), PMA (1 µM; 0.5 h; C and D) and BaCl₂ (1 mM; 0.5 h; E and F) treated cells were fixed, immunostained with the MNK (1:1000; A, C and E) and PHM (1:1000; B, D, and F) antibodies and then incubated with FITC-conjugated rabbit IgG antibody. All cells were observed by epifluorescence microscopy. The scale bar equals 10 µm (F). Nucleus (N). Wide arrows (TGN/perinuclear region), arrowheads (secretory granules) and double thin arrows (vesicular structures).
PHM protein was localized to distinct punctate vesicles or secretory granules (Fig. 55B, D, and F; thin arrows and arrowheads). Thus, in the AtT-20 PAM-1 cells (Fig. 56), the localization of the MNK protein cannot be altered by secretagogues, unlike in the AtT-20 WT cells.

**Analyses of Expression of MNK in Response to PMA and BaCl$_2$ in AtT-20 Cells**

Since we were able to alter the localization of MNK in AtT-20 WT cells following treatment with PMA, the next step was to examine if MNK expression changes in response to secretagogues. Thus, AtT-20 WT and PAM-1 cells were stimulated as described in Fig. 53. To determine if immature and mature secretory granules underwent fusion with the plasma membrane and secreted their contents into the medium in response to either PMA or BaCl$_2$, the medium from control and treated cells was collected, concentrated, fractionated by SDS-PAGE and Western blot analyses performed using the PC1 or PHM antibodies (Fig. 57A and B). The PC1 antibody was used to determine the secretion of granular contents in the AtT-20 WT cells, as these cells do not express PAM, and to confirm that secretion was stimulated. As is typical of stimulation experiments, in the first and second basal medium collections, PC1 was only detected as faint bands at 80 and 60 kDa in the control cells that received medium for either 30 or 120 min period (Fig. 57A, B1 and B2). The difference in the amount of PC1 detected in B1 and B2 between the experiments reflects normal differences in the basal secretion of PC1. Following the stimulation period in the AtT-20 WT control cells with medium not containing secretagogues, an increase in PC1 was not detected after the 30 or 120 min periods (Fig. 57A; control panels; S30’ and S120’). In the PMA treated cells, the 80 kDa band of PC1 remained unchanged in the first and second basal medium collections and from the stimulated media (Fig.
Figure 56: Stimulation of AtT-20 PAM-1 Cells with PMA and BaCl$_2$

Control AtT-20 PAM-1 Cells

A.  
B.  

MNK Ab  
PHM Ab

PMA Stimulated AtT-20 PAM-1 Cells

C.  
D.  

MNK Ab  
PHM Ab

BaCl$_2$ Stimulated AtT-20 PAM-1 Cells

E.  
F.  

MNK Ab  
PHM Ab

Figure 56: Stimulation of AtT-20 PAM-1 Cells with PMA and BaCl$_2$

Control (A and B), PMA (1 µM; 0.5 h; C and D) and BaCl$_2$ (1 mM; 0.5 h; E and F) treated cells were fixed, immunostained with the MNK (1:1000; A, C and E) and PHM (1:1000; B, D, and F) antibodies and then incubated with FITC-conjugated rabbit IgG antibody. All cells were observed by epifluorescence microscopy. The scale bar equals 10 µm (F). Wide arrows (TGN/perinuclear region) and arrowheads (punctate staining or secretory granules). Nucleus (N).
Figure 57: Analyses of Collected Medium from AtT-20 WT and PAM-1 Cells Stimulated with BaCl$_2$ and PMA. AtT-20 cells were incubated with PMA (1 µM) or BaCl$_2$ (1 mM) for 30 (S30’) or 120 (S120’) min. Media from the 1$^{st}$ basal (B1), 2$^{nd}$ basal (B2) and stimulation (S) periods were collected, fractionated by SDS-PAGE and probed by immunoblotting for PC1 in the WT cells (A) and PHM in the PAM-1 cells (B).
while an increase in the secreted amount of the 60 kDa band of PC1 was observed in the stimulated medium from both the 30 and 120 min treatment periods (Fig. 57; PMA panel; S30’ and S120’). Lastly, in the medium from the 30 or 120 min BaCl2 treated cells, a significant increase in the 60 kDa band of PC1 was observed (Fig. 57A; BaCl2 panels; S30’ and S120’). Overall, these data indicate that our secretagogues were able to cause the exocytosis of the 60 kDa PC1 form contained within secretory granules.

In the media collected from the AtT-20 PAM-1 control and treated cells as described in Figure 52, the PHM antibody was used to confirm that secretory granule exocytosis had occurred and the PHM protein had been secreted into the medium. In the control AtT-20 PAM-1 cells, the PHM protein primarily was detected as a 45 kDa band in the basal media (Fig. 57B; B1 and B2), with only a slight difference in the amount observed between the samples (Fig. 57B; control panels; S30’ and S120’). For the PAM-1 cells treated with PMA, an increased level of the PHM protein (45 kDa) was observed in the stimulated medium following both the 30 and 120 min treatments as expected (Fig. 57B; PMA panel). Finally, in the BaCl2 treated cells, an increase in PHM secretion was observed in the 30 and 120 min stimulated medium (Fig. 57B; BaCl2 panels; S30’ and S120’). In comparison, slightly more of the secreted PHM protein was detected in both the first basal and stimulated media from the AtT-20 PAM-1 cells treated with BaCl2 for 120 min when compared to the 30 min treatment (Fig. 57B; BaCl2 panels; S30’ and S120’).

Next, the AtT-20 WT and PAM-1 cell extracts obtained as described in Figure 52 were analyzed for the presence of MNK, PC1 and PHM proteins. Samples were fractionated by SDS-PAGE and Western blot analyses were performed. For the control, 30 and 120 min treated AtT-20 WT cells, the MNK protein was detected as a 178 kDa band with the MNK antibody (Fig 58A; MNK Ab panels). In the AtT-20 control cells that received medium without secretagogues
AtT-20 Wild Type Cell Extracts

30 min Stim | 120 min Stim

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<tr>
<th>Cont</th>
<th>PMA</th>
<th>BaCl₂</th>
<th>Cont</th>
<th>PMA</th>
<th>BaCl₂</th>
</tr>
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</table>

**A.**

- **MNK Ab**
  - 178 kDa

- **PC1 Ab**
  - 80 kDa
  - 60 kDa

- **Tubulin Ab**
  - 55 kDa

AtT-20 PAM-1 Cell Extracts

30 min Stim | 120 min Stim

<table>
<thead>
<tr>
<th>Cont</th>
<th>PMA</th>
<th>BaCl₂</th>
<th>Cont</th>
<th>PMA</th>
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**B.**

- **MNK Ab**
  - 178 kDa

- **PHM 1764 Ab**
  - 120 kDa
  - 105 kDa
  - 45 kDa

- **Tubulin Ab**
  - 55 kDa

Figure 58: Expression of the MNK Protein in AtT-20 WT and PAM-1 Cells Stimulated with Secretagogues. AtT-20 WT (A) and PAM-1 (B) cells were cultured for four days prior to treatment with PMA (1 µM; 30 or 120 min Stim), or BaCl₂ (1 mM, 30 or 120 min Stim). Control cells received only medium during the stimulation period (Cont). 25 µg of sample was loaded onto a 10% gel and fractionated by SDS-PAGE. Western blot analyses were performed using the following antibodies: MNK (1:1000), PC1 (1:1000), PHM (1:1000) and tubulin (1:500).
for either 30 or 120 min, the detected levels of the MNK protein were similar. Interestingly, in
AtT-20 WT cells treated with PMA for 30 min, the MNK protein detected from the cell extracts
was not significantly different than that of the control cells (Fig. 58A; 30 min PMA), while more
of the MNK protein was detected in the cells treated with PMA for 120 min as compared to the
120 min control (Fig. 58A; 120 min PMA). In the AtT-20 WT cells treated with BaCl₂ for 30
and 120 min, MNK protein levels were less than that of the control and PMA treated cells (Fig
58A; BaCl₂, MNK Ab panels). As opposed to the data shown in Fig. 57, the PC1 antibody was
used for the cell extracts to determine if PC1 protein levels decreased, with increasing
stimulation times. In the control 30 and 120 min treated cells, PC1 was detected as 80 and 60
kDa bands (Fig. 58A; PC1 Ab panels). In both the PMA and BaCl₂ treated cells, the 80 and 60
kDa bands were detected at similar levels as in the control cells, with a greater decrease in both
forms of PC1 in cells treated with BaCl₂ for 120 min observed (Fig 58A; PC1 Ab panels). The
decrease mainly occurring for the 60 kDa PC1 form agrees with this form being detected at
higher amounts from the stimulated medium from these cells. Lastly, the tubulin antibody was
used as an internal control of protein loading and the tubulin protein was detected (55 kDa) in the
cell extracts (Fig. 58A; tubulin). Overall, these results indicate that the secretagogues were able
to promote exocytosis of vesicles containing the PHM and PC1 proteins in the AtT-20 cells.

For comparison and to determine if the presence of the PAM protein affects the
expression of the MNK protein following exposure of the AtT-20 PAM-1 cells to PMA and
BaCl₂, these cells also were examined as described in Figure 52. In the 30 and 120 min control
and stimulated cells, the expression of the MNK protein as a 178 kDa band was observed using
the MNK antibody (Fig. 58B; MNK Ab panels). In cells treated with PMA or BaCl₂, the MNK
protein was expressed at lesser levels as compared to the control cells (Fig. 58B; MNK Ab left
panel), while the levels of the MNK protein detected following the 120 min stimulation periods with both secretagogues remained unchanged (Fig. 58B; MNK Ab right panel).

In the AtT-20 PAM-1 cells, we used the PHM antibody to determine if the PHM protein levels decreased following increased stimulation of secretory granule exocytosis. In these experiments, the PAM-1 protein was detected as a 120 kDa band and the PHM protein was observed as a 45 kDa band in the control, 30 and 120 min PMA and BaCl₂ treated cells (Fig 58B; PHM Ab panels). Notably, the PAM-1 and PHM protein expression levels were similar (Fig. 58B; PHM Ab panel). In addition, a 105 kDa band, representing the soluble PAM-1 protein was detected in the cell extracts from the PMA and BaCl₂ treated cells (Fig. 58B; PMA and BaCl₂ panels). Finally, as for the AtT-20 WT cells, the tubulin protein was used as an internal control of protein loading and detected in AtT-20 PAM-1 control and treated cell extracts as a 55 kDa band (Fig. 58B; tubulin Ab panel).

**Analyses of Expression of MNK Responses to Various Secretagogues in AtT-20 Cells**

Stimulation experiments followed by Western blot analyses as detailed in Figure 53 were performed to examine the expression of the MNK protein in response to a variety of secretagogues (PMA, BaCl₂, cAMP, and CRH) in AtT-20 WT and PAM-1 cells. In the WT cells, a 178 kDa band of the MNK protein was detected with the MNK antibody in the control and treated cell extracts (Fig 59A; MNK Ab panel). Moreover, in cells treated with PMA or CRH for 1 h, there was an increase in the level of the MNK protein detected as compared to the controls, while in the BaCl₂ and cAMP treated cells, only a slight increase in MNK protein expression was observed as compared to the controls following stimulation (Fig. 59A; MNK Ab panel). As stated previously, we used the PC1 antibody to determine if the PC1 protein levels
Figure 59: Expression of the MNK Protein in AtT-20 WT and PAM-1 Cells Stimulated with Secretagogues. AtT-20 WT (A) and PAM-1 (B) cells were cultured for four days prior to treatment with PMA (1 μM; 1 h), BaCl₂ (1 mM, 1 h), cAMP (1 μM; 1 h), or CRH (1 μM; 1 h). Control cells received only medium during the stimulation period. 30 μg of protein from each sample was loaded onto 10% gels and fractionated by SDS-PAGE. Western blot analyses were performed using the following antibodies: MNK (1:1000), PC1 (1:1000), PHM (1:1000) and tubulin (1:500).
decreased in cell extracts following secretagogue stimulation. In the control, PMA, BaCl₂, cAMP, and CRH treated cells, the PC1 protein was detected as 80 and 60 kDa bands (Fig. 59A; PC1 Ab panel). In the PMA and BaCl₂ treated cells, there was a slight decrease in the PC1 protein as compared to the control cells; however, there was not a concomitant decrease in the expression of the PC1 protein in the cAMP and CRH stimulated cells (Fig. 59A; PC1 Ab panel). As for the previous experiments, the tubulin protein (55 kDa) was used as an internal control of protein loading.

MNK protein expression in response to the aforementioned stimulators also was examined in the AtT-20 PAM-1 cells. Unlike in the WT cells, however, MNK protein expression appeared to be similar between the control, PMA, BaCl₂, cAMP, and CRH stimulated cells (Fig. 59B; MNK Ab panel), especially if variances in sample loading were taken into account based on the detection of the tubulin protein (55 kDa) as an internal loading control (Fig. 59B; tubulin Ab panel). Furthermore, the detection of the PAM-1 (120 kDa) and PHM (45 kDa) proteins, as potential indicators of secretory granule exocytosis, was similar in the control and treated cells (Fig. 59B; PHM Ab panel). In general, our data indicate that the expression of the MNK protein is regulated by a potentially large number of intracellular signaling molecules.
Discussion

In primary anterior pituitary and AtT-20 cells, the regulated secretory pathway has been well studied. Previous studies have shown that the addition of the secretagogues, PMA, CRH, and BaCl₂, to cultured pituitary and AtT-20 cells, stimulated the secretion of their secretory granule contents (El Meskini et al., 2000; El Meskini et al., 2001; Ferraro et al., 2005). Furthermore, in media collected from PMA, BaCl₂ and CRH stimulated rat anterior pituitary cultured cells, an increase in the secretion of the PHM, PAM-3, sPAM-2, and sPAM-1 proteins has been observed when compared to control medium. In addition, in these stimulated cells, an increase in PHM protein activity also has been observed (El Meskini et al., 2000). Since PAM protein function is partially dependent on the delivery of copper by the MNK protein, it is possible that the MNK protein could be regulated in response to the same secretagogues. Therefore, we wanted to examine the effect of secretagogues on MNK trafficking and protein expression in AtT-20 WT and PAM-1 cells.

To do this, we used two general secretagogues, BaCl₂ and PMA, which are known to stimulate exocytosis in endocrine cells, to determine if the MNK protein ever resides in secretory granules in AtT-20 cells (Von Ruden et al., 1993). BaCl₂, which acts like the second messenger calcium, inactivates potassium channels, resulting in the depolarization and opening of calcium gated channels. This, in turn, causes the fusion of secretory granules with the plasma membrane as well as the release of the soluble contents of mature secretory granules (Von Ruden et al., 1993). The phorbol ester, PMA, acts on protein kinase C by mimicking the effects of diacylglycerol to cause the secretion of immature and mature secretory granules (Billiard et al., 1997). For example, it has been shown in PMA, BaCl₂ or both PMA/BaCl₂ stimulated rat primary anterior pituitary cultured cells that the secretion of GH increased as compared to the
control cells. Furthermore, immunocytochemistry experiments demonstrated that GH showed a
different localization pattern in stimulated cells from that in control cells (Ferraro et al., 2005).

To determine the effects of BaCl₂ and PMA on the localization of the MNK, PC1 and
PHM proteins, immunofluorescence microscopy was performed on non-stimulated and
stimulated AtT-20 WT and PAM-1 cells. In the AtT-20 WT cells, an increase of the MNK
protein in punctate vesicles distributed along the plasma membrane was observed primarily in
the PMA treated cells. The presence of these punctate structures only in cells stimulated with
PMA rather than BaCl₂ can be explained by the fact that two distinct populations of secretory
granules (immature and mature) are targeted by PMA, while BaCl₂ only stimulates exocytosis
from mature granules. The MNK protein staining pattern in the PMA stimulated cells also is
similar to the one observed for the PC1 protein, indicating that the MNK protein is present in
stimulatable granules. In the AtT-20 PAM-1 cells, the MNK protein was localized to the
perinuclear region in untreated cells and its localization remained the same in the PMA and
BaCl₂ treated cells. In contrast, the secretory granule protein, PHM, was localized to punctate
vesicular structures along the plasma membrane following stimulation of the AtT-20 PAM-1
cells. Interestingly, the staining pattern of the MNK protein differed between the AtT-20 WT
and PAM-1 cells, indicating that in the presence of the PAM-1 protein, there is less MNK protein
in vesicular structures than after stimulation with PMA and BaCl₂.

To further examine the effects of PMA and BaCl₂ on the localization and expression of
the MNK, PAM and PC1 proteins, stimulation experiments followed by Western blot analyses
were performed. The levels of two secretory granule proteins, PC1 (60 kDa) in WT and PHM
(45 kDa) in PAM-1 cells, both increased in the medium from cells treated for 30 or 120 min with
PMA or BaCl₂ when compared to medium from the control cells (Fig. 57), indicating exocytosis
of secretory granules. A decrease of the PC1 protein in the cell extracts from cells stimulated with PMA and BaCl₂ for 30 or 120 min also was observed. The decrease of the PC1 protein in the cell extracts following stimulation is in agreement with the PC1 protein being secreted into the medium (Figure 58). Although the MNK protein was observed in vesicular structures at the plasma membrane in PMA treated cells, MNK protein secretion was not stimulated; thus, reflecting the membrane bound topology of the MNK protein.

In the PMA and BaCl₂ treated AtT-20 PAM-1 cells, no changes in PAM-1 protein expression levels were observed as compared to control cells, while the PHM protein was only slightly increased in the treated cells. However, after the 30 min stimulation of the AtT-20 WT cells with PMA and BaCl₂, MNK protein levels (178 kDa) were decreased when compared to control cells (Fig. 58). Interestingly, MNK protein expression levels increased following the 120 min treatment with PMA, while stimulation with BaCl₂ for the same length of time produced a slight decrease in MNK protein expression. Furthermore, in the AtT-20 PAM-1 cells, a slight decrease in MNK protein expression following the 30 min stimulation with PMA or BaCl₂ also was observed with a significant change observed after the 120 min treatment. These results indicate that MNK protein expression can be affected by PMA in AtT-20 cells.

The effects of two other secretagogues, CRH and cAMP, on the expression of the MNK, PAM and PC1 proteins also were analyzed. Corticotropes are the only cell type in the pituitary that can be stimulated by CRH to secrete ACTH (Ooi et al., 2004). Previous experiments have determined that the CRH stimulation of anterior pituitary cells can produce an increase in PAM protein cleavage products in medium (El Meskini et al., 2000). The signaling molecule, cAMP, has been shown to act on protein kinase A, leading to the phosphorylation of downstream proteins. Since the majority of the protein kinases that phosphorylate the MNK protein have not
been determined, it is possible that MNK protein phosphorylation by protein kinase A following its activation by cAMP could alter MNK protein activity.

Western blot analyses of the MNK protein from the AtT-20 WT cells showed an increase in MNK protein expression (178 kDa) in cells treated with cAMP and CRH, while no significant changes in PC1 protein expression (80 and 60 kDa) were observed. In addition, in the PAM-1 cells, no significant differences in MNK and PHM (45 kDa) protein expression levels between control and treated cells were observed. The results obtained from the media collected from the AtT-20 PAM-1 cells stimulated with PMA, BaCl₂ and CRH were comparable to the results obtained in previous studies (El Meskini et al., 2000; El Meskini et al., 2001; Ferraro et al., 2005). In general, these data suggest that cAMP and CRH may play a regulatory role on the expression of the MNK protein in AtT-20 cells.

The results from these studies indicate that even though both cell types can undergo exocytosis, in WT cells, MNK protein expression and localization are more readily altered by treatment with secretagogues than in PAM-1 cells, where MNK protein expression and localization remains constant. Although both cell lines express endogenous levels of the MNK protein, the extra copies of the PAM protein present in the PAM-1 cells could have an effect on the localization and regulation of the MNK protein in response to secretagogues. Since MNK mRNA was not examined, it is not clear whether the observed effects of secretagogues on MNK protein expression play a transcriptional or translational regulatory role.

In summary, this study examined the effects of stimulating secretory granule exocytosis on the localization and expression of the MNK protein in AtT-20 cells. It is clear from these studies that some of the secretagogues such as PMA, CRH and cAMP can affect the regulated trafficking, localization and expression of the MNK protein in the AtT-20 WT and PAM-1 cells.
However, it will be necessary to develop further studies to investigate the regulation of MNK protein expression as well as the possibility that the MNK and PAM proteins have a unique interaction in the secretory compartments of these cells.
Conclusions

Dietary copper is a necessary element for organisms and serves as a cofactor for several enzymatic reactions. To ensure the proper copper balance, organisms express copper chaperones and the P-type ATPase, Menkes copper transporter (MNK). Mutations within the MNK gene result in the X-linked genetic disorder known as Menkes disease. This disease is characterized by neurodegeneration, connective tissue defects, hair abnormalities, hypopigmentation, and early childhood death. Symptoms in patients with Menkes disease are attributed to a deficiency in the delivery of copper to cuproenzymes, including peptidylglycine α-amidating monooxygenase (PAM) and cytochrome c oxidase.

Previous studies have examined the trafficking and localization of MNK in fibroblasts transfected with MNK in response to copper levels. However, MNK localization and trafficking have not been closely examined in endocrine cells containing endogenous MNK and relevant cuproenzymes such as PAM. Our studies using immunofluorescence microscopy, subcellular fractionation, and Western blot analyses demonstrated that MNK was localized to the TGN and secretory granules in AtT-20 WT and PAM-1 cells at steady-state. In cells exposed to high copper levels, MNK was localized to the TGN as well as to diffuse punctate vesicular structures in the cytoplasm, while in low levels of copper, MNK remained in the TGN. These studies presented relevant and new information regarding a differential localization of MNK to secretory granules in endocrine cells. Since many proteins use signals in specific domains to regulate and direct their trafficking, these findings will allow us to develop further studies to investigate the routing determinants of MNK important for MNK sorting and trafficking in endocrine cells.

Since mutations in the MNK protein can lead to altered localization of the protein in the cell and the failure to deliver copper to enzymes, a comparison between the trafficking of the WT MNK
and mutated MNK will provide us with essential information about MNK function in endocrine
cells in relationship to the cuproenzyme PAM.

Although the expression of MNK varies in different tissues from rats, mice and humans,
and since MNK has been shown to deliver copper to the cuproenzyme, PAM, studies were
performed to explore the possibility that MNK and PAM are coordinately regulated during
development. In these studies, MNK expression was examined at postnatal day 3, day 30 and
adult in several brain tissues such as the cortex, cerebellum, medulla, hypothalamus and
hippocampus, as well as endocrine tissues such as the pituitary, adrenal glands, and heart from
Sprague-Dawley rats. Our results indicate that when PAM expression is compared to MNK
expression, some coordinated regulation was observed in the adrenal glands, where an increase
in both MNK and PHM was observed and in the hippocampus where a decrease in MNK and
PHM was observed. Surprisingly, in the pituitary, MNK protein levels decreased, while sPAM-2
levels increased with the animal’s age. These results suggest that the expression of both MNK
and PAM proteins is tissue specific and that there is little similarity of the expression of MNK
and PAM proteins between the brain and endocrine tissues. Therefore, further studies will be
developed to gain a better understanding of the regulation of MNK expression during
development in endocrine and brain tissues, with the ultimate goal of determining the best age to
administer copper to patients with MNK disease in hopes of alleviating some of the debilitating
symptoms associated with this disease.

Previous studies have shown that the treatment of primary pituitary and AtT-20 cells with
secretagogues such as PMA, CRH and BaCl2 stimulate the secretion of secretory granule
proteins including PAM and also altered PAM expression. Since PAM activity depends on the
delivery of copper by MNK, we examined the effect of secretagogues on MNK localization and
protein expression in AtT-20 cells. Our studies showed that MNK was localized to the TGN as well as punctate vesicular structures at the plasma membrane following treatment with PMA, indicating that MNK was found at the plasma membrane as a result of fusion of secretory granules during exocytosis. Interestingly, MNK expression also was increased after treatment with PMA, CRH and cAMP, while BaCl₂ slightly decreased in its expression. Since changes in MNK expression can affect copper delivery and as a result regulate the activity of cuproenzymes, it is essential to understand the mechanisms involved in regulating MNK expression. Therefore, further studies will be performed to determine if MNK expression is regulated at the transcriptional and/or translational level(s) as well as its relationship to the regulation of the cuproenzyme, PAM. These studies will allow us to gain a better understanding of the adjustments endocrine cells undergo in response to physiological stimuli.

In summary, MNK expression and localization in endocrine cells differs from that of fibroblasts transfected with MNK. These studies provide significant preliminary information aimed at investigating MNK function in endocrine cells. Since the localization, trafficking and regulation of MNK seems more complex than originally thought, the sorting and regulatory mechanisms for MNK function need to be further explored to better understand neurodegenerative diseases such as Menkes disease.
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