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The Characterization of Menkes Copper Transporter and Dopamine β-monooxygenase Carboxy-Terminus in Neuroendocrine Cells

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The Characterization of Dopamine β-Monoxygenase Carboxy-Terminus and Menkes Copper Transporter in Neuroendocrine Cells

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DEDICATION

I would like to dedicate this work to my parents, V. Rev. Joseph and Elen Antypas, who have been most supportive and encouraging in the pursuit of my education. It is through their love and sacrifice that I have been able to realize my dreams.
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CHAPTER I: INTRODUCTION (Part 1)

Synthesis and release of catecholamines are key events in the response of organisms to a variety of stimuli including stress (Nessler, 1997; Nessler, 1999; Valentino et al., 1998; Aston-Jones et al., 2000). Dopamine $\beta$-monooxygenase (DBM) is an essential enzyme that catalyzes the synthesis of norepinephrine from dopamine, in a hydroxylation reaction that requires molecular oxygen, copper, and reduced ascorbate as cofactors (Partoens et al., 1988; Stewart and Klinman, 1988; Merkler et al., 1995; Tanner et al., 1996). DBM is only one of its kind amongst the catecholamines biosynthetic enzymes in its subcellular localization because it is the only enzyme in this biosynthetic pathway located in the secretory granule lumen of adrenal chromaffin cells and adrenergic neurons (Tanner et al., 1986; Winkler et al., 1986; Stewart and Klinman, 1988; Winkler, 1993; Oyarce and Eipper, 2000).

It has been shown that the structure of DBM consists of a signal sequence, a pro-region, a putative catalytic domain, and a COOH-terminal domain as indicated by amino acid sequences in rat, mouse, bovine, and human (Lamouroux et al., 1987; Kobayashi et al., 1989; Taljanidisz et al., 1989; Lewis, 1990; McMahon et al., 1990; Nakano et al., 1992;). Each DBM monomer contains 15 cysteine residues that form six intramolecular and two interchain disulfide bridges (Robertson et al., 1994). DBM contains six N-glycosylation sites where three of them are conserved in human DBM (McMahon et al., 1990). Furthermore, DBM monomers form disulfide-linked dimmers that associate
noncovalently to originate the tetrameric DBM protein (Sabban et al., 1983; Saxena and Fleming, 1983; Saxena and Hensley, 1985; Robertson et al., 1994). The putative catalytic domain in DBM has been identified based on its 32% homology to the catalytic core of the peptidylglycine \( \alpha \)-hydroxylating monooxygenase (PHM) domain of the amidating enzyme, peptidylglycine \( \alpha \)-amidating monooxygenase (PAM) (McMahon et al., 1990). Interestingly, the PHM domain also requires molecular oxygen, copper and reduced ascorbate as cofactors (Southan and Kruse, 1989).

It has been well established that DBM is found in both soluble and membrane-bound forms within secretory granules (Sabban et al., 1983; Saxena and Fleming, 1983; Winkler et al., 1986). Furthermore, both forms of DBM are derived from a single mRNA (Lamouroux et al., 1987; Stewart and Klinnman, 1988; Bon et al., 1991; Lewis and Asnani, 1992; Tanner et al., 1996). It is important to point out that the soluble DBM is released along with catecholamines upon stimulation of exocytosis of chromaffin cells and neurons (Ito, 1983), while membrane-bound DBM fuses with the plasma membrane from where it is retrieved by endocytosis (Partoens et al., 1988; Hurtley, 1993). Although the structural and biosynthetic relationship between both forms of DBM is not completely understood, studies have shown that the signal sequence of DBM is retained in 30% of the membranous DBM suggesting a possible role as a membrane anchor due to a hydrophobic region near its NH\(_2\)-terminus (Stewart and Klinman, 1989; Taljanidisz, 1989). Furthermore, recent studies where the signal sequence of DBM was appended to the PHM domain to form the
DBMsignal/PHMs chimera indicate that retention of the DBM signal sequence confers a type II membrane topology to the chimera and averts its access to the secretory granules in AtT-20 cells (Oyarce et al., 2001). Since DBM contains a putative catalytic core, it is possible that the domains flanking the catalytic core play a role in DBM trafficking and/or maintaining the structural feature of the enzyme. Because little is known about the COOH-terminal domain of DBM (DBMCT), we aimed at investigating the role of DBMCT in sorting and trafficking of DBM. Since it is difficult to study the role of DBMCT in the full length DBM protein due to its large tetrameric structure and glycosylation, the DBMCT containing a rhodopsin tag was attached to the reporter PHM domain of the amidating enzyme to create the PHMs/DBMCT chimera (PHMs/DBMCT). The PHMs/DBMCT chimera was expressed and characterized in AtT-20 cells, a tumor cell line derived from corticotropes of the anterior pituitary, which contain regulated secretory granules. The behavior of the PHMs/DBMCT protein was compared to that of PHM expressed in AtT-20 cells. PHM was selected as a reporter protein because is has homology to the DBM catalytic core, requires the same cofactors as DBM for activity and its function and trafficking has been extensively studied (Southan and Kruse, 1989; McMahon et al., 1990; Eipper et al., 1993).

The subcellular localization of the active PHMs/DBMCT chimera in AtT-20 cells was analyzed by immunofluorescence microscopy and subcellular fractionation into secreted, soluble, and membranous fractions. Additional studies of the membranous fractions were performed by carbonate wash
treatment to analyze the membrane association of the chimera. The biosynthesis and secretion of PHMs/DBMCT was studied using metabolic labeling and stimulation of AtT-20 cells with the secretagogue, BaCl$_2$, while proteolytic cleavage of the chimeric protein was analyzed by Western blot using the rhodopsin and PHM antibodies. Since our data showed that the chimera is sorted to secretory granules and undergoes proteolytic cleavage in AtT-20 cells, further studies were carried out to determine whether or not this cleavage occurs in the full length DBM. For these studies, DBM containing a myc tag was stably transfected into PC12 cells and analyzed by Western blot. PC12 cells are a tumor cell line derived from the adrenal medulla that synthesize catecholamines and contain a regulated secretory pathway. PC12 cells were used in these studies because it has been shown that full length DBM sorts to secretory granules in these cells, while it does not traffick to secretory granules in AtT-20 cells due to cell-type specificity of sorting and storage (Oyarce and Eipper, 2000).

Because PHM, when expressed in AtT-20 cells, sorts to secretory granules, it was difficult to determine whether the PHMs/DBMCT localization in secretory granules was a result of sorting information present in the DBMCT domain or if it was the sorting and trafficking information present in the PHM domain was exerting its effect on the chimera. Thus, it was important to isolate DBMCT and study this domain without the possible effects of PHM by appending the DBMCT to the carboxy-terminus of the green fluorescent protein, EGFP, to generate the EGFP/DBMCT chimera. EGFP is a protein that was isolated from the pacific jellyfish and is used as a reporter for gene expression by acting as a
fluorescent protein tag for protein localization (Davenport and Nicol, 1955). This chimera was then transiently expressed in PC12 cells and live cells were analyzed by fluorescence microscopy. Because EGFP only acts as a reporter tag and contains no intrinsic trafficking properties, this approach allows us to isolate the sorting information in the DBMCT by analyzing the behavior of the chimeric protein.
CHAPTER I: INTRODUCTION (Part 2)

Copper is an essential element that is required for all organisms to survive (Harris, 2003). This important metal has the capability to take on distinct redox states where it can be oxidized as Cu\(^{2+}\) or reduced to Cu\(^{1+}\) form in order to participate in chemical reactions and serve as a cofactor for several cuproenzymes (Hartman and Evenson, 1992). For example, the effects of copper deficiency in Cytochrome c oxidase, which is important in electron transport in cellular respiration, results in ATP shortage, myopathy, ataxia, and seizures (Sparaco et al., 1993). In addition, abnormal lysyl oxidase results in connective tissue disorders and vascular rupture (Kodama et al., 1999), while proper functions of Angiogenin and Clotting Factor V are important in blood vessel formation and blood clotting (Yang, 2006). Similarly, copper deficiency also affect both DBM and PAM, which are cuproenzymes involved in catecholamine biosynthesis and post-translational amidation of bioactive peptides, respectively. Studies have shown that copper deficiency disrupts DBM function in embryonic development and catecholamine systems resulting in hypothermia, hypotension, and dehydration (Hartman and Evenson, 1992; Pena et al., 1999), while its effects on PAM result in impaired peptide amidation (Steveson et al., 2003). Malfunction of PAM and DBM has a significant effect in the synthesis of active peptide hormones and catecholamines leading to widespread multi-systemic effects (Pena et al., 1999; Petris et al., 2000; Mercer, 2001).
Although it is known that copper is vital to an organism’s survival, its redox nature can cause cellular toxicity when excess levels of copper are present (Pena et al., 1999; Waggoner et al., 1999; Loudianos and Gitlin, 2000). Due to the paradoxical phenomena of copper being essential, and yet toxic, its distribution must be precisely regulated by copper chaperones and transporters in order to maintain cellular homeostasis (Suzuki and Gitlin, 1999; Mercer, 2001; Markossian and Kurganow, 2003). Regulation of copper homeostasis in mammals is essential, as demonstrated by genetic disorders of copper deficiency and copper toxicity (Bremmer, 1988; Sparaco, 1993; Kaler, 1994; Kaler et al., 1998; Harrison and Dameron, 1999; Pena et al., 1999; Loudianos and Gitlin, 2000). Disorders of copper deficiency include the lethal Menkes disease and the two milder disorders called Occipital Horn Syndrome and mild Menkes disease (Menkes, 1962; Qi and Byers, 1998). Menkes disease is an X-linked recessive copper deficiency syndrome that affects approximately 1 in 300,000 individuals (Tumer and Horn, 1998). Studies have shown that the disease is caused by an allelic mutation of ATP7A gene encoding the Menkes protein (MNK) and its locus has been mapped to Xq13.3 with the gene having been isolated by positional cloning (Mercer et al., 1993). It is important to point out that in Menkes disease, the cellular copper uptake is normal, while its efflux from the intestinal epithelial mucosal cells is affected, disrupting the delivery of copper to essential cuproenzymes (Mercer, 2001). Interestingly, the Menkes disease which is a multi-systemic disorder of defective copper utilization by essential cuproenzymes
is characterized by steely hair, hypopigmentation, mental retardation, neurodegeneration, abnormal myelination, convulsions, failure to grow, and early childhood death (Hartmann and Evenson, 1992; Harrison and Dameron, 1999; Pena et al., 1999; Waggoner et al., 1999).

Expressed in most tissues, except the liver, MNK is a copper transporting P-type ATPase (Vulpe et al., 1993) that transplants copper across the basolateral membrane of mucosal cells (Harrison and Dameron, 1999) into the bloodstream through an energy-dependent mechanism requiring ATP. Interestingly, MNK functions through a covalent phosphorylated enzyme intermediate formed when the γ-phosphate of ATP reacts with an aspartic acid residue in a conserved DKTG sequence forming a putative channel (Pedersen and Carafoli, 1987). MNK has a molecular weight of 178 kDa and contains six putative heavy metal binding GMXCXXC motifs at its N-terminus (Yamaguchi et al., 1996). It has been suggested that this highly conserved consensus sequence binds copper in its reduced Cu^{+} form to transport it across biological membranes (Hasset and Kosman, 1995).

Studies using MNK transfected fibroblasts show that MNK is primarily localized to the trans-golgi network (TGN) (Suzuki and Gitlin, 1999). However, under elevated copper conditions MNK moves to the plasma membrane from the TGN to return copper to normal levels (Petris et al., 1996), while normal levels or depletion of copper returns MNK to the TGN (Petris et al., 1996; Petris and Mercer, 1999). Although trafficking of MNK in response to copper levels have been examined in fibroblasts expressing exogenous MNK, little is known about
the subcellular localization and trafficking of MNK in neuroendocrine tissues that endogenously express MNK and cuproenzymes. The chromaffin cells of the adrenal gland is a good system to study the subcellular localization and function of MNK because they contain the cuproenzyme DBM, synthesize catecholamines, and endogenously express MNK. More importantly, the adrenal chromaffin cells contain regulated secretory granules where catecholamines and peptides are stored; these organelles are not present in fibroblasts. In addition to chromaffin cells, in our studies we also used the PC12 cell line that is a tumor cell derived from the adrenal medulla because it has the same characteristics of the adrenal chromaffin cells.

To evaluate the role of MNK in the catecholamine system in a patient with Menkes disease, it is essential to understand the localization and function of endogenous MNK in endocrine cells. We hypothesize that MNK has a differential subcellular distribution in neuroendocrine cells. Therefore, the second part of this thesis project was aimed at characterizing the endogenous MNK protein in adrenal chromaffin and PC12 cells. In order to determine the subcellular localization of MNK, adrenal chromaffin and PC12 cells were subjected to differential fractionation and sucrose density gradient fractionation couple to Western blot analysis and immunoflourescence microscopy. To further confirm the subcellular localization of MNK in neuroendocrine cells, we also analyzed the trafficking of MNK in response to copper levels, as well as following stimulation with a secretagogue. For these studies, PC12 cells were incubated with copper or a copper chelator to stimulate a copper-dependent trafficking to
the plasma membrane and/or TGN. To analyze the copper-independent trafficking of MNK, PC12 cells were incubated with high concentrations of KCl to induce exocytosis of secretory granules. Then, localization of MNK was determined by cell surface biotinylation, immunofluorescence microscopy, and Western blot analysis.
CHAPTER II: LITERATURE REVIEW (Part 1)

The synthesis and release of catecholamines is essential for a variety of normal and stress-induced homeostatic mechanisms (Nessler, 1997; Nessler, 1999; Ressler and Nemeroff, 1999; Valentino et al., 1998; Aston-Jones et al., 2000). Organisms survive by maintaining homeostasis and stress-induced responses that are mediated by catecholamines are essential to preserve the body’s dynamic equilibrium. Therefore, divergence in the function of these important chemical compounds play a major role in a variety of maladies (Cryer, 1980). For instance, it has been shown that orthostatic intolerance, a disorder that shows symptoms of orthostatic tachycardia, palpitations, dizziness, and fatigue is due to raised plasma levels of norepinephrine (Robertson, 1986). This disorder, which usually affects women between the ages of 20-50 years, is characterized by too much sympathetic activation in response to stimuli. On the other hand, orthostatic hypotension is a disorder characterized by faint levels of norepinephrine and epinephrine, and elevated levels of dopamine (Robertson, 1986). These patients have a lack of the norepinephrine-synthesizing enzyme dopamine β-monooxygenase and exhibit a decrease in autonomic and cardiovascular function. Clearly, these various studies suggest that an understanding of catecholamines and their biosynthetic pathway have important implications in human disease.
Catecholamine Biosynthetic Pathway

Catecholamines are synthesized in the adrenal medullary chromaffin cells and in sympathetic ganglia and nerves (Blaschko, 1939). These compounds include dopamine, norepinephrine, and epinephrine and are synthesized from the
amino acid L-tyrosine in a pathway that uses the enzymes tyrosine hydroxylase, dopa decarboxylase, dopamine β-monooxygenase, and phenylethanolamine N-methyltransferase (Flatmark et al., 2002; Parmer and Zinder, 2002) (Fig. 1).

In this pathway, Tyrosine hydroxylase is responsible for the conversion of L-Tyrosine to 3,4 dihyroxy-L-phenylalanine (L-DOPA) and requires Fe(II), tetrahydrobiopterin, and molecular oxygen to function (Blaschko, 1939). In neuroendocrine cells, this enzyme exists in a soluble and membrane bound form and is considered the rate-limiting step in the catecholamine biosynthetic pathway (Kuhn and Yoon, 1990). Following the formation of L-DOPA, the enzyme L-DOPA decarboxylase that requires pyridoxal 5-phosphate (vitamin B6) as a cofactor in order to function (Moore et al., 1996), catalyzes the decarboxylation of L-DOPA to form dopamine (Blaschko, 1939). This enzyme not only catalyzes the above reaction, but also decarboxylates all other occurring aromatic L-amino acids and is generally called L-aromatic amino acid decarboxylase.

The conversion of dopamine to norepinephrine is catalyzed by the enzyme dopamine β-monooxygenase (DBM) that requires copper, ascorbate, and molecular oxygen in order to function (Friedman and Kaufman, 1965; Terland and Flatmark, 1975; Abudu et al., 1998). DBM is unique amongst the catecholamine biosynthetic enzymes in its subcellular localization because it is the only enzyme in this biosynthetic pathway located in the secretory granule.
Figure 2. Dopamine is converted to norepinephrine by DBM in a reaction that requires copper, ascorbate, and molecular oxygen. Semihydroascorbate is a byproduct of this hydroxylating reaction.

The lumen of adrenal chromaffin cells and adrenergic neurons (Tanner et al., 1986; Winkler et al., 1986; Winkler, 1993; Stewart and Klinman, 1988; Oyarce and Eipper, 2000). The synthesis of norepinephrine is very important because this crucial neurotransmitter in the central and peripheral nervous system plays a significant role in regulating mood, attention, arousal, and cardiovascular function while being an important modulator in other physiological functions (Nestler and Aghajanian, 1999). Interestingly, it has been reported that human norepinephrine deficiency is associated with mutations in the DBM gene, rendering a protein nonfunctional (Kim and Zabetian, 2002). In the last step of this biosynthetic pathway, norepinephrine undergoes a methylation reaction that is catalyzed by the enzyme phenylethanolamine N-methyl transferase to form epinephrine (Wong and Yamasaki, 1987). The synthesis of epinephrine occurs primarily in the adrenal medulla chromaffin cells, while studies have shown that this enzyme is localized in the cytosol and that the methyl donor S-
adenosylmethionine is required for the methylation reaction to take place (Grunewald et al., 1988). Once epinephrine is synthesized, it is stored in secretory granules, along with norepinephrine, until the cell is stimulated for their release.

Catecholamines together with soluble proteins are released from the adrenal medulla and adrenergic and noradrenergic neurons via exocytosis (Ito, 1983). The signaling cascade that is responsible for their release involves acetylcholine-mediated depolarization of the cell leading to calcium influx (Burgoyne, 1984). Once the cells have been stimulated, the secretory granules move from the cytoplasm to fuse with the plasma membrane allowing their soluble contents to be released into the extracellular space (Laduron, 1975). Studies have showed that the secretory granules are accumulated beneath the plasma membrane in a process known as “docking” and that it is the calcium influx through voltage-gated calcium channels that induces the fusion of the granules to the plasma membrane (Augustine and Neher, 1992). While significantly more work needs to be done to understand the mechanism by which calcium is able to trigger granule fusion and catecholamine release, studies have identified proteins on synaptic vesicles and plasma membrane that are involved in this process (Augustine, 2001). The ATPase NSF (NEM-sensitive fusion protein) and SNAPs (soluble NSF-attachment proteins), which are important in vesicle-golgi interactions, are thought to be important in priming synaptic vesicles for fusion (Sudhof, 1995). These proteins work to regulate the assembly of another class of proteins called SNAREs (SNAP receptors) to form the SNARE
complex that allows for the attachment of the vesicle to the plasma membrane. One of these SNARE proteins, synaptobrevin, is localized to the membrane of secretory vesicles whereas syntaxin and SNAP-25, also members of the SNARE protein class, are found on the plasma membrane (Sudhof, 1995). Additionally, it is hypothesized that synaptotagmin, which is not a member of the SNARE proteins, plays an important role in exocytosis by acting as a calcium sensor which triggers vesicle fusion (Tucker and Chapman, 2002). Although the exact mechanism by which binding of calcium to synaptotagmin leads to exocytosis remains unclear, it is thought that the protein undergoes a conformational change allowing for SNARE complex formation (Augustine, 2001).

Just as there is a mechanism by which secretory granules are able to undergo exocytosis for release of their contents, it is known that a mechanism also exists where granules that have undergone exocytosis can be retrieved from the plasma membrane by endocytosis. Endocytosis of secretory granules is clathrin-mediated and occurs at a slower rate than exocytosis (Henkel and Almers, 1996). It is thought that phosphoinositides (PI), activitated by specific PI kinases such as Arf-GTP kinase, play a role in mediating this process whereby phosphoinositides control the timing and localization of endocytic trafficking by recruiting adaptors, such as AP-2, to the transport machinery. Synaptotagmin proteins, namely synaptotagmin 1 and 3 interact with these adaptors at the plasma membrane allowing for the formation and function of the clathrin coated pits (Koenig and Ikeda, 1996). In endocytosis, the clathrin coat protein mediates invagination of the plasma membrane which is followed by membrane scission, a
required step to release the coated vesicle. Once the vesicle is internalized, it undergoes an uncoating step catalyzed by an “uncoating ATPase” followed by its interaction with the endosomal compartment giving rise to new secretory vesicles (Heuser and Reese, 1973). While this explanation gives some insight into the how endocytosis occurs, the precise molecular mechanism of this process remains unclear.

**The Adrenal Chromaffin Granule**

The biochemical composition of the chromaffin granules consists of the catecholamines dopamine, norepinephrine, and epinephrine, in addition to nucleotides, ascorbic acid, calcium and other proteins and peptides such as enkephalins and neuropeptide Y (Winkler et al., 1986; Schultzberg et al., 2005). In addition to these peptides, the major component of the chromaffin granule are the chromogranins A, B, and C which function in sorting of regulated secretory proteins to granules (Blaschko et al., 1967; Winkler et al., 1986). It has been shown that a member of this class of proteins, chromogranin A, is an “on/off” switch that is sufficient to turn on secretory granule biogenesis and hormone sequestration in endocrine cells (Kim et al., 2001). A study conducted by Kim et al. (2001) showed that a downregulation of chromogranin A expression by antisense RNA led to a loss of secretory granules and impaired regulated secretion. Most importantly, transfection of the chromogranin A protein into a non-endocrine fibroblast cell line CV-1 induced the production of secretory
granules. Interestingly, chromogranins B and C was not observed to play a role in secretory granule biogenesis (Kim et al., 2001).

The adrenal chromaffin granule, in addition to their soluble components, also contain several membrane proteins. It has been shown that a $V_o$-ATPase, a proton-pump responsible for maintaining the acidic pH of secretory granules, is present in the granule membrane (Cidon and Nelson, 1983; Johnson, 1988). Furthermore, the protein cytochrome $b_{561}$, which functions in electron transport and has the important function of regenerating ascorbate for DBM function (Kent and Fleming, 1987), is also found embedded in the granule membrane (Silsand and Flatmark, 1974). In addition, the vesicle monoamine translocase is another membrane-bound protein that functions as a catecholamine transporter across the granule membrane. This protein has Lastly, two cuproenzymes DBM and peptidylglycine $\alpha$-amidating monooxygenase (PAM) are also found rooted in the secretory granule membrane. These proteins will be discussed in detail below.

**Dopamine $\beta$-monooxygenase (DBM)**

DBM is the only catecholamine biosynthetic enzyme localized to the secretory vesicles (Tanner et al., 1986; Winkler et al., 1986; Stewart and Klinman, 1988; Winkler, 1993; Oyarce and Eipper, 2000) that catalyzes the conversion of dopamine to norepinephrine (Friedman and Kaufman, 1965; Terland and Flatmark, 1975; Abudu et al., 1998) by using molecular oxygen, copper, and ascorbate as cofactors, this enzyme. Ascorbate present in high
concentration in secretory granules is the molecule that acts as an electron donor in this mechanism (Friedman and Kaufman, 1965; Terland and Flatmark, 1975). The first step in the DBM reaction involves the oxidation of ascorbate to semidehydroascorbate. Ascorbate is then regenerated by the shuttling of electrons via protein cytochrome b$_{561}$ (Srivastava, 1995). As ascorbate becomes oxidized, it reduces the enzyme bound copper (II) to copper (I) allowing the reduced DBM enzyme to react with molecular oxygen to form water and, in turn, cause the hydroxylation of its dopamine (Friedman and Kaufman, 1965).

DBM exists as a tetrameric glycoprotein of 290 kDa that is held together via disulfide linkages and non-covalent interactions (Lewis and Asnani, 1992; Sabban et al., 1983; Saxena and Fleming, 1983). Each DBM monomer contains 15 cysteine residues, forming seven disulfide bridges and six N-glycosylation sites (Robertson et al., 1994). DBM monomers form two disulfide-linked dimers that associate noncovalently to originate the tetrameric DBM protein (Sabban et al., 1983; Saxena and Fleming, 1983; Saxena and Hensley, 1985; Robertson et al., 1994). Although DBM exists as a tetrameric species, it has been shown that this enzyme can undergo a reversible dissociation of its subunits that is pH dependent (Saxena and Hensley, 1985).

Within the secretory granule, the DBM enzyme exists in soluble and membrane bound forms, both of which have been shown to derive from a single translational product (Winkler et al., 1986; Lamouroux and Vigny, 1987; Stewart and Klinman, 1988; Taljanidisz et al., 1989; Bon et al., 1991). Pulse-chase experiments carried out by Sabban et al. (1982) in pheochromocytoma (PC12)
cells showed that the 77 kDa form of DBM is initially synthesized and processed to a 73 kDa form within 15-90 minutes, thus suggesting that the membranous DBM precedes and originates the soluble form (Sabban et al., 1982). However, this data does not support the processing of membranous DBM to the soluble form because both forms are synthesized at a similar rate. Furthermore, no decrease in the 77 kDa form was observed at any chase time. Although both forms of DBM undergo glycosylation, the role of glycosylation of DBM has not been well understood. However, when tunicamycin, a drug that functions as an inhibitor of glycosylation, was used to treat PC12 cells, much of the non-glycosylated form is degraded suggesting that glycosylation of DBM may be important in lending stability to the protein (Sabban et al., 1982).

The amino acid sequences of DBM from rat, mouse, bovine, and human indicate that the DBM structure consists of a signal sequence, a pro-region, a putative catalytic domain, and a carboxy-terminal domain (Fig. 3) (Lamouroux and Vigny, 1987; Kobayashi et al., 1989; Taljanidisz et al., 1989; Lewis et al., 1990; McMahon et al., 1990; Nakano et al., 1992). Although the structural and biosynthetic relationship between both forms of DBM is not completely understood, studies have suggested that DBM exists as a type II integral membrane protein (Feng and Angeletti, 1992). Moreover, in studies developed to elucidate the role of the DBM signal sequence, the DBM signal sequence was appended to the reporter protein peptidyl-glycine α-hydroxylating monooxygenase (PHM), a soluble protein domain of the peptidylglycineα-amidating monooxygenase (PAM) protein to originate the DBM signal/PHMs
Figure 3. DBM structure and topology

A.

B.

Figure 3. A.) The structure of monomeric DBM consists of a signal sequence, pro-region, catalytic domain, and COOH-terminal domain. There are multiple cysteine residues present throughout the protein, several of which participate in intramolecular disulfide bridges. Oligosaccharides are indicated by the lollipop sticks. B.) Membrane topology of DBM is shown in the lumen of the secretory granule. DBM tetramer is formed by non-covalent interaction of dimmers by disulfide-linked monomers. It has been suggesting that DBM is tightly associated to the membrane by its signal sequence.

chimera. Interestingly, subcellular fractionation on cells expressing the DBMsignal/PHMs chimera showed that the chimera was found in the membrane fraction whereas PHMs alone was found in the soluble fraction (Oyarce et al., 2001). Most importantly, the topology of the chimera indicates that the signal
sequence contain information to anchor DBM to secretory granule membrane with a type II topology. This finding is in agreement with the DBM signal sequence being the only hydrophobic domain in DBM (Sabban et al., 1983).

Since DBM belongs to a family of enzymes known as monooxygenases, it shares homology with other enzymes such as PAM and MON X (Husten and Eipper, 1991; Eipper et al., 1992; Eipper et al., 1993). PAM is an essential enzyme that amidates over 50% of bioactive peptides at the peptide's carboxy terminus (Eipper and Mains, 1980). Peptides that require this amidation reaction include substance P, neuropeptide Y, vasoactive intestinal peptide, thyroid releasing hormone and other peptides that are generated from proopiomelanocortin (POMC) processing by proteolytic processing (Eipper, 1993; El Meskini et al., 2000). PAM carries out the amidation by converting peptidylglycine substrates into α-amidated products in two steps: the first step involves a hydroxylating reaction by PHM while the second step involves a lyase reaction via peptidyl-α-hydroxyglycine α-amidating lyase (PAL) domain (Eipper, 1993). The PHM reaction is the rate limiting step of the amidating reaction and requires copper, ascorbate, and molecular oxygen (Eipper et al., 1992) in order to catalyze the formation of peptidyl α-hydroxyglycine intermediates (Eipper, 1993; Prigge et al., 1999). The PAL reaction requires zinc and completes the amidating reaction by catalyzing the conversion of this intermediate into the amidated product, a reaction primarily occurs in the secretory granules (Eipper, 1993). The structure of PAM consists of several domains that includes the signal
Figure 4. The PAM-1 protein is a bifunctional enzyme that consists of a pro-region, PHM hydroxylating domain, exon A, PAL lyase domain, a transmembrane domain (TMD), and a cytosolic domain (CD). The putative catalytic core was identified based on its 32% homology to the PHM domain of PAM.

sequence and pro-region (Fig. 4), followed by the hydroxylase domain (PHM) and the lyase domain (PAL). PAM also has a COOH-terminus, a transmembrane domain (TMD) that is essential to anchor PAM to secretory granule membrane with a type I topology. It has been shown that the PAL lyase domain contains the only N-glycosylation site in PAM protein. The PAM gene is able to undergo alternative splicing to yield soluble and membrane bound forms of the enzyme and there are at least seven different forms of this protein (Eipper, 1993). In PAM-1, the longest form of PAM, PHM and PAL domains are separated from each other by a segment, exon 16, which has no catalytic properties but does have an endoproteolytic cleavage site. Because there are many similarities between DBM and PAM, the putative catalytic domain of DBM
has been identified based on its 32% homology to the catalytic core of the PHM domain of the PAM bifunctional enzyme (Fig. 4) (Southan and Kruse, 1989). The sequence similarities between these two proteins point to the fact that they are evolutionarily related by evolving from a common precursor.

Since DBM contains a putative catalytic core, it is possible that the domains flanking the catalytic core play a role in DBM sorting and trafficking and/or maintaining the structural feature of the enzyme. The COOH-terminal domain of DBM (DBMCT) contains two cysteine residues (Fig. 1; Cys\textsuperscript{531} and Cys\textsuperscript{533}) involved in the formation of interchain disulfide bridges between two monomers to originate DBM dimmers. In addition, DBMCT also contains two Cys residues involved in intrachain disulfide bonds and two of the six potential glycosylation sites present in DBM (Robertson et al., 1994). The fact that DBM is a large tetrameric glycoprotein makes it difficult to evaluate the role of the DBMCT in the native protein. Therefore, in this thesis, we aim at evaluating the role of the DBMCT in sorting and trafficking of DBM. For these studies, DBMCT was appended to the PHM domain of PAM. PHM was used as a reporter protein because it has similarities to DBM (Fig. 4) and its behavior has been well characterized (Eipper and Mains, 1980; Eipper et al., 1992; Eipper, 1993).
Copper is an essential element that is required for organisms, from bacteria to humans, for survival (Harris, 2000). Copper ions have the ability to adopt distinct redox states, oxidized as $\text{Cu}^{2+}$ or reduced to $\text{Cu}^{+}$ form. Thus, the ability to participate in redox chemical reactions allows copper to serve as an important catalytic cofactor. By acting as a prosthetic group, copper permits the transfer of electrons to many enzymes that carry out essential biological tasks (Pena et al., 1999; Waggoner et al., 1999).

There are many enzymes that require copper as a cofactor in order to function in growth and development (Hartmann and Evenson, 1992). These enzymes include cytochrome c oxidase, superoxide dismutase and lysyl oxidase, amongst many others. Cytochrome c oxidase is a component of the mitochondrial electron transport chain, superoxide dismutase serves as a defense against free radicals and lysyl oxidase is an enzyme important for the cross-linking of collagen and elastin. Without copper playing its role in the reactions of these key enzymes, they would be rendered non-functional, resulting in brain abnormalities, hypothermia, muscle weakness, connective tissue defects, or a sensitivity to oxidative stress (Harris, 2000; Mercer, 2001). Other imperative enzymes that require copper are those that are crucial for brain function such as PAM that amidates over 50% of all neuropeptides, and is essential in the modification of various neurotransmitters (Prigge et al., 2000) and DBM that is crucial in the catecholamine synthesis pathway in which
norepinephrine is synthesized from dopamine (Friedman and Kaufman, 1965). In a study conducted by Prohaska and Brokate (1999) it was reported that DBM mRNA and activity were altered in male Holtzman rats given a copper-deficient diet and low copper drinking water for 4 weeks. When catecholamine levels were measured in the adrenal gland from copper-deficient rats lower norepinephrine levels, while higher dopamine levels were observed. Thus, an impair function of these important cuproenzymes, severe neurological effects, such as mental retardation, seizures, feeding difficulties, and decreased muscle tone, could result (Hartman and Evenson, 1992; Strausak et al., 2001).

Although it is known that copper is vital to an organism’s survival, it can also be highly toxic when accumulated in excess. Excessive copper can take part in the generation of highly reactive oxygen species and can displace other metal cofactors from their natural ligands (Harrison and Dameron, 1999). Furthermore, copper overexposure can lead to the development of liver cirrhosis, hemolysis, damage to renal tubules and brain, and other organs (Pena et al., 1999; Waggoner et al., 1999). Interestingly, the symptoms of copper toxicity are not constant and can vary between species (Bremmer, 1998). For example, it has been reported that in pigs, copper toxicity usually gives rise to weakness, respiratory distress, anemia, and jaundice, while in rats, excessive copper intake manifests itself in growth impairment and hepatic necrosis (Bremmer, 1998). Due to the paradoxical phenomena of copper being essential and yet toxic, the distribution of this metal must be precisely regulated in order to maintain cell homeostasis.
Contributing to the goal of maintaining copper homeostasis are specific copper transporters and a class of proteins called copper chaperones (Camakaris et al., 1999; Harris, 2000). Copper transporters and copper chaperones serve this function by mediating cellular copper import/export and function to deliver copper to specific places within the cell. For example, it has been shown that copper is transported across the plasma membrane and into the cell via the human copper transporter protein (hCTR-1) (Klomp et al., 2002; Lee et al., 2002). The CTR transporter, which was first identified in yeast Saccharomyces cerevisiae, is analogous with the human hCTR-1 and is essential for copper entry into the cell (Lee et al., 2002). Studies using hCTR-1 infected into Sf9 insect cells and have shown that the protein is localized to the plasma membrane where it is involved in increasing the rate of copper uptake by 20-30 fold compared to control Sf9 cells (Eisses and Kaplan, 2002). In a separate study, it was shown that hCTR-1 transports copper into human embryonic kidney cells (HEK 292) in a time-dependent manner (Lee et al., 2002). These data clearly indicate the role that hCTR-1 plays in copper uptake in the cell.

Once in the cell, copper is bound to copper chaperones for distribution to subcellular organelles. These chaperones, also originally identified in yeast, bind copper and deliver it them to cuproenzymes or to other copper transporters for delivery to cuproenzymes (Markossian and Kurganow, 2003). Specific copper chaperones include Hah-1, hCox 17, CCS, and Atx1. hCox 17 delivers copper to the mitochondria for incorporation into cytochrome C oxidase and CCS.
delivers copper to the Cu/Zn superoxide dismutase in the cytosol. Atx1 delivers copper to the golgi and mitochondria for incorporation into multicopper ferroxidase, Fet 3, while it is Hah-1 that brings copper to the Menkes protein ATP7A ATPase (MNK) in the TGN. The ability of Hah-1 to shuttle copper to MNK allows it to undergo an energy driven transport of copper across organelle membranes for incorporation into cuproenzymes (Markossian and Kurganow, 2003).

Expressed in all tissues, except the liver, MNK is the main copper exporter across the basolateral membrane of mucosal cells (Mercer, J. F. 2001). Dietary copper intake for adults is usually between 0.6-1.6 mg/day and most comes from food sources that are rich in copper such as seafood, organ meats, nuts, and legumes (Mercer, 2001). Copper is absorbed in the small intestines and is first transported through the brush-border of mucosal cells through the apical membrane. Once in the mucosal cells, copper is either sequestered by metallothionein or is bound by Hah-1 and exported into the bloodstream through the basolateral membrane via the Menkes copper transporter (Mercer, 2001). Once in the bloodstream, copper is bound to serum proteins, such as albumin and ceruloplasmin, and taken to various tissues for incorporation into cuproenzymes. Because efflux of copper from the basolateral membrane of intestinal epithelial cells into the bloodstream occurs by an energy-dependent mechanism, requiring ATP, the MNK protein is a P-type ATPase (Vulpe et al., 1993) and is the rate-limiting step in copper absorption (Harrison and Dameron, 1999).
Figure 5. Copper is ingested via dietary sources and is absorbed through enterocytes of the small intestines where it is then pumped into the vasculature by MNK. Copper is metabolized in the liver and then transported to target organs for incorporation into cuproenzymes. Excess copper is excreted in bile and eliminated in the feces.

The precise mechanism by which the MNK ATPase functions to transport copper across biological membranes has been extensively studied (Vulpe et al., 1993). In order to pump copper across the cell membrane, the energy of ATP hydrolysis is needed. This reaction that proceeds through a covalent phosphorylated enzyme intermediate that is formed when the γ-phosphate of ATP reacts with a single aspartic acid residue in the conserved DKTG sequence of the ATPase, resulting in a conformational change and ion transport. This step is then immediately followed by a dephosphorylation step where the phosphate is
hydrolyzed to restart the cycle. The sequence of phosphorylation and dephosphorylation steps is what grants the energy for copper to be transported across the membrane (Pederson and Carafoli, 1993). Although there are differences amongst the members of the P-type ATPase family, these transporters also share general features and have several regions of high homology (Lutsenko and Kaplan, 1995). For instance, the MNK gene is organized into 23 exons spanning a genomic region of approximately 150 kb and encodes an ATPase of 1500 amino acids with a molecular weight of 178 kDa (Dierick et al., 1995). MNK contains several domains that are conserved amongst the P-type ATPases (Fig. 6). For example, MNK contains eight transmembrane domains and its structure contains an ATP binding motif (GDGXNG), the phosphorylation motif (DKTG), a phosphatase motif (TGES), and a CPC motif which is involved in ATP hydrolysis and copper transport. The MNK ATPase also contains six heavy metal binding (CXXC) motifs at its N-terminus, a GDGNVD “hinge” motif, and an LL motif which functions in internalization of the protein (Petris et al., 1998). It is the highly conserved CXXC motif that serves to bind copper in its reduced Cu^{1+} form for transport across biological membranes (Hasset and Kosman, 1995).

Homologous to MNK is the Wilsons protein ATP7B (WND). This protein is also a member of the P-Type ATPase family, has a molecular mass of 165 kDa and contains the same structural elements required for catalysis than MNK. Therefore, the amino acid sequence motifs that are important in MNK function is conserved in WND. For example, The GMXCXXC heavy metal binding motif,
Figure 6. Menkes copper transporter ATPase

Figure 6. The MNK ATPase has eight transmembrane domains and several different motifs as indicated by the color legend. The copper binding domains are found at the NH$_2$ terminus while the dileucine motif is found at the COOH-terminal. However, both the NH$_2$ and COOH-terminus domains are in the cytosol. Figure is courtesy of Dr. Tami Steveson, Bowling Green State University, Bowling Green, Ohio.

The TGES phosphatase motif, DKTG phosphorylation motif, and the GDGXNG ATP binding motif are all found in both of these copper transporters (Schaefer and Gitlin, 1999). Interestingly, it has been reported that WND is to be localized to the TGN region in HepG2 cells by sucrose density gradient experiments and immunofluorescence microscopy (Suzuki and Gitlin, 1999). WND is mainly expressed in the liver where it functions to transport copper into the secretory pathway where it binds to ceruloplasmin and is then excreted into bile (Fig. 5) (Loudianos and Gitlin, 2000). Mutations in WND render a non-functional protein and the development of Wilson’s disease. Because this ATPase is expressed in
hepatocytes and is the sole mechanism for copper excretion, patients with Wilson's disease suffer from a progressive accumulation of copper in the liver. When the capacity for copper storage in the liver is exceeded, cell death ensues and copper is released into the plasma and deposited in tissues resulting in liver toxicity and neuropsychiatric symptoms (Loudianos and Gitlin, 2000). For example, patients may present with acute liver failure or may have a chronic active hepatitis which most commonly culminates in liver cirrhosis. Neuropsychiatric symptoms include early dementia, mood disorders or personality changes, dystonia, and tremor secondary to copper accumulation in the central nervous system (basal ganglia, putamen, or globus pallidus). Other symptoms found in Wilson’s disease may be renal tubular acidosis, copper deposition of the iris (Kayser-Fleicher rings), cardiomyopathy or arrhythmia, and hemolysis of red blood cells (Schilsky, 2005).

Like the WND protein, studies have shown MNK is also localized to the TGN region in HeLa fibroblasts by confocal microscopy (Yamaguchi et al., 1996). Similar results were observed examining MNK localization in other cell lines. For example, in the human breast carcinoma cell line, PMC42, and in MNK transfected Chinese hamster ovary cells (CHO), immunofluorescence studies showed a perinuclear staining indicative of TGN (Ackland et al., 1997; Strausak et al., 1999). Several studies have been developed in an effort to identify what region of the MNK protein contains the trafficking information required to route MNK to the TGN. Using MNK constructs, Francis et al. (1998) identified a TGN localization signal in the transmembrane domains 3 and 4.
Using immunofluorescence microscopy, the construct encoding the full length MNK co-localized with TGN-46, a resident protein of the golgi, while MNK lacking exon 10 was found in the endoplasmic reticulum (ER) with resident protein calnexin. Additionally, using CD8, a resident protein of the plasma membrane, as a reporter molecule, Francis et al. showed that the attachment of a segment of exon 10 encoding transmembrane domain 3 relocalized CD8 from the plasma membrane to the TGN (Francis et al., 1998). Clearly, these data demonstrate that transmembrane domain 3 of MNK contains routing information for MNK to reside in the TGN.

While it is apparent that in transfected fibroblasts MNK is found in the TGN, it is also observed that under elevated copper conditions MNK moves to the plasma membrane for copper efflux (LaFontaine and Mercer, 2007) and bring copper levels to a normal physiological state (Strausak et al., 1999). Thus, this rapid trafficking of MNK causes a change in its subcellular localization and steady-state distribution in response to copper levels (Petris et al., 1996; Petris and Mercer, 1999). A copper-resistant variant of CHO fibroblasts, which have MNK gene amplification and consequently overexpress MNK, was utilized to examine how MNK responds to changes in copper levels (Petris et al., 1996). It was observed by immunofluorescence that MNK localized to the TGN when copper-resistant cells were incubated in media containing basal levels of copper. However, when cells were incubated in excess copper, MNK rapidly trafficked to the plasma membrane co-localizing with the cell membrane marker, Na⁺/K⁺ ATPase. Furthermore, the steady-state distribution of MNK was reversible and
not dependent on the new synthesis of MNK protein as evidenced by the fact that the amount of MNK mRNA and protein was not affected by changes in copper concentration (Petris et al., 1996). Clearly, this data indicates that MNK trafficking to the plasma membrane functions to regulate copper efflux and maintain homeostasis. Similar findings were observed in HeLa cells and human breast carcinoma PMC42 cells (Ackland et al., 1997; Suzuki and Gitlin, 1999).

In the case of the WND protein, it is apparent that it too undergoes a copper-dependent trafficking under elevated copper conditions (LaFontaine and Mercer, 2007). The mechanism of regulation of copper export by WND has become evident from similar studies showing the subcellular distribution of this protein in response to copper levels (de Bie et al., 2007). For instance, in HepG2 liver cells, WND is localized to the TGN under steady-state conditions, while it trafficks to the plasma membrane under conditions of copper excess. This observation is consistent with its function in excretion of copper from the hepatocyte via the bile (Schaefer et al., 1999).

Much work has been done in order to better understand the mechanism by which MNK trafficks in response to excess copper and what domains of the MNK protein are responsible for this action. As mentioned previously, MNK is able to transport copper through the formation of an acyl-phosphate intermediate. While this phosphorylation motif (CPC) of MNK is necessary for its catalytic activity, it has been shown that this region also plays an important role in MNK ability to move in response to increasing copper levels (Petris et al., 2002). In this studies, MNK mutants containing mutations in the conserved CPC motif and
phosphatase motif of MNK (TGES) were transfected in CHO fibroblasts in order to analyze MNK trafficking. It was observed that when the formation of the phosphorylated intermediate was hindered due to the mutation in the CPC copper transduction domain, increased copper levels could not cause MNK to traffic from the TGN to the plasma membrane. Moreover, when the phosphatase domain (TGES) was mutated to induced hyperphosphorylation, MNK underwent a constitutive trafficking to the plasma membrane from the TGN independent of copper, suggesting that copper-induced relocalization depends on the MNK ATPase catalytic cycle initiate MNK trafficking. A similar phenomena was observed when the phosphatase domain of WND transporter was mutated (Petris et al., 2002). Therefore, while mutations in this region would render the protein unable to transport copper across membranes, this suggests that these domains are also important in the ability of MNK to re-localize in response to copper.

While mutations in the CPC and phosphatase domains of MNK serve to disrupt normal MNK behavior in response to elevated copper conditions, studies performed by Strausak et al. have also implicated the GMXCXXC copper binding motifs as also important in MNK trafficking. Following site-directed mutagenesis of the six copper metal binding sites (MBS) where cysteine residues were changed to serine residues, different mutated MNK constructs were transfected into CHO cells and MNK trafficking in response to 300 μM copper was analyzed (Strausak et al., 1999). Immunofluorescence microscopy showed that when MBS 1, MBS 6 or MBS 1-3 were mutated, MNK redistributed to the plasma
membrane in response to excess copper. However, when MBS 4-6 or MBS 1-6 were mutated, MNK did not re-localize from the TGN to the plasma membrane. Similar findings were observed when only MBS 5 or 6 were mutated. Furthermore, N-terminal deletions of the first four MBS did not impair MNK trafficking to the plasma membrane suggesting that due to their close proximity to the cation transporting channel, MBS 5 or MBS 6 is necessary and sufficient for MNK trafficking in response to increasing copper levels (Strausak et al., 1999).

The fact that there exists a mechanism by which MNK trafficks to the TGN to the plasma membrane, studies have shown that MNK is retrieved from the plasma membrane back to the TGN once the cell reaches copper homeostasis (Francis et al., 1999; Lane et al., 2004). Deletions of different regions of the MNK cytoplasmic domain were appended to the CD8 reporter protein in order to identify a motif responsible for MNK retrieval from the plasma membrane. The chimeric proteins were transiently transfected into MRC5/V2 human fibroblasts and analyzed by immunofluorescence. Wild-type cells expressing only CD8 showed staining at the plasma membrane, while cells expressing the CD8 chimera containing only the last 62 amino acids of the MNK cytoplasmic domain showed a more punctate staining with very little staining at the plasma membrane. The punctate staining corresponds to the staining of the chimera in endosomes indicating that an internalization motif was present in this 62 amino acid sequence of the cytoplasmic domain. Interestingly, three di-leucine motifs (LL) and a tyrosine motif (YSRA) that are been known to play a role in internalization of proteins were found in the 62 amino acid sequence of the MNK
cytoplasmic domain (Francis et al., 1999). Site-directed mutagenesis studies
determined that the di-leucine motif L\textsuperscript{1487}L\textsuperscript{1488} is required for endocytosis of MNK
and its mutation results in abnormal MNK behavior with mislocalization and
impairment of MNK internalization (Francis et al., 1999).

**Menkes Disease**

Menkes disease (MD) is a fatal disorder that is caused by mutations in
the gene encoding the ATP7A copper transporting P-type ATPase (Bertini and
Rosato, 2008), involved in the regulation of intracellular copper levels through
efflux and delivery of copper to cuproenzymes. Menkes disease was first
identified in 1962 by Dr. John Menkes (Menkes et al., 1962) and it is
characterized by hypothermia, thrombosis, alterations in bone, arterial defects,
steely hair, hypopigmentation, odd face, and severe neurological defects which
include mental retardation, abnormal myelination, convulsions, failure to grow,
and early childhood death (Harrison and Dameron, 1999; Kodama et al., 1999;
Waggoner et al., 1999). Interestingly, the disease presents itself as a multi-
systemic disorder and affects approximately 1 in 300,000 individuals and its
locus has been mapped to the X chromosome at q13.3, thus primarily affecting
males (Kaler, 1994). Patients with this disease usually die before three years of
age and it is reported that it is very rare that a patient be diagnosed in the
neonatal period (Kodama et al., 1999). More commonly, patients seem to be
normal up until two or three months of age before the symptom onset begins.
When a patient presents with MD, the caregiver typically has noticed a loss of developmental milestones, observed profound truncal hypotonia or complains that their child has recurrent seizures (Bahi-Buisson et al., 2006). In fact, clinical manifestation of seizures in MD patients were classified by Bahi-Buisson et al. (2006) and they occur in three stages. The early stage, occurs at a median age of 3 months and is characterized by focal clonic seizures; while at a median age of 10 months, patients typically develop intractable infantile spasms which characterizes the intermediate stage. In the last stage, that occurs around 25 months of age, a patient will have multifocal seizures, tonic spasms and myoclonus. Thus, as the patient gets older, they are subject to increasing neurological complications.

Physical exam findings of patients with MD demonstrate kinky, short, twisted or course hair that is lightly or abnormally pigmented and abnormal facies revealing jowly sagging cheeks and ears, a depressed nasal ridge and a high arch palate. Ocular manifestations include ptosis, visual inattention, and iris hypopigmentation, while bone abnormalities comprise multiple congenital fractures and osteoporosis (Gasch et al., 1999; Kodama et al., 1999). Laboratory tests that can aid in the diagnosis of MD consist of a serum copper < 70 mg/dL (normal= 80-160 mg/dL), decreased plasma norepinephrine and increased DOPA, and hypoglycemia while magnetic resonance imaging (MRI) may demonstrate white matter demyelination and cerebral atrophy (Geller et al., 1999; Kaler et al., 1993). Furthermore, magnetic resonance angiography (MRA) may reveal tortuous blood vessels giving a corkscrew like appearance and due to
vascular malformation typical in patients with MD, subdural hematomas are common as well as stroke in those that survive longer, as detected by computerized tomography (Geller et al., 1999).

Mutation to the ATP7A gene can give rise to MD; however, depending on where this mutation arises, a milder form of the disease has been reported (Kaler, 1998). Occipital Horn Syndrome (OHS) is described as the mild form of MD and is caused by mutation in the splice site of exon 10 leading to a deletion of this exon (Qi and Byers, 1998). It is reported that this mutation leads to an in-frame deletion of transmembrane domains 3 and 4, eliminating the golgi localization signal, causing the MNK protein to be localized to the ER in patients with OHS (Qi and Byers, 1998). OHS is characterized by some of the symptoms of MD, but are much less severe. Both MD and OHS have the characteristic kinky steely hair and facial features; however, the neurological deficits in OHS are not as profound as these patients may have mild mental retardation and autonomic dysfunction. The predominant features of OHS are the connective tissue and skeletal anomalies that are seen such as hyperelastic and bruisable skin and hyperextensible joints. However, the characteristic feature of this disease is the occipital exostoses, or “horns”, which are calcifications that are found in the occipital tendinous insertions of the trapezius and sternocleidomastoid muscles (Kaler, 1995; Tumer and Horn, 1998; Qi and Byers, 1998)

Unlike Wilson’s disease (WD), which shows symptoms of abnormal liver function due to elevated copper leading to toxicity, MD shows very low levels of
copper in the liver, but a very high amount in the intestines (LaFontaine and Mercer, 2007). Because mutations in the MNK protein affect cellular copper efflux from intestinal mucosa into the blood stream, it is observed that MD patients have low serum copper levels and thus many of the cuproenzymes cannot receive copper for their normal function (Kodama et al., 1999). A study conducted by Sparaco et al. (1993) found that the expression cytochrome c oxidase (COX), a cuproenzyme located in the mitochondrial inner membrane and involved in the production of ATP, was deficient in a patient with MD. Analysis of COX protein in cerebellum, spinal cord, and other areas of the central nervous system of a 13-month old patient that died of MD showed a reduced expression of COX. Thus, it is plausible that the COX deficiency found in a patient with MD may contribute to symptoms of this disorder by playing a pathogenic role in the neuronal degeneration of a MD patient.

Just as a deficiency in COX has been reported in MD patients, studies aimed at investigating the effect on the cuproenzyme DBM have also been conducted (Kaler et al., 1993; Strausak et al., 2001). It has been shown that patients with MD have abnormal catechol levels in serum and cerebrospinal fluid (CSF) (Kaler et al., 1993). Assaying plasma and CSF fluid samples that were taken from patients with MD showed that in comparison to a control group, the MD group displayed higher L-DOPA and dopamine levels, while the levels of NE were significantly lower in the MD group. Due to the fact that DBM is the only enzyme responsible for the conversion of dopamine to norepinephrine in secretory granules of adrenergic and noradrenergic neurons, this suggests that
DBM function is impaired in MD patients and may be a contributing factor to the manifestation of the many neurological symptoms observed in MD patients.

While many studies have been done on patients with MD, another approach to studying this disease involves using a mouse model. Although several mouse models exist to study the MNK protein, the most commonly used are the mottled-brindled (Mo-Br) and mottled-blotchy (Mo-Bl) mouse models. The Mo-Br is a mouse model that most closely resembles the human Menkes disease, while the Mo-Bl is a closer representation to the milder form, OHS (Cecchi and Avner, 1996). These mice have a mutation in the MNK gene impairing copper transport and therefore, the activity of many cuproenzymes are affected (Cecchi and Avner, 1996; Hunt, 1976). Because the polypeptide sequence of MNK in the human and mouse are well conserved, the abnormalities seen in these mice resemble the features of MD patients, including depigmentation, curly hair, skeletal abnormalities, and defective elastin and collagen (Cecchi and Avner, 1996). Menkes mottled mice have been used in many studies in order to elucidate the regulation of copper in cells that do not have a functional MNK (Packman, 1987; LaFontaine et al., 1999). In one such study, La Fontaine et al. (1999) investigated the basis for the brindled and blotchy phenotypes of these mice by examining the intracellular distribution of the Mnk protein, the murine homologue of MNK, and its copper transporting characteristics, in cultured cells from these mutant mice. Fibroblasts from brindled and blotchy mice were incubated these cells in radiolabeled copper for 2, 4, and 16 h accumulated significantly more copper over the 16 hour period.
compared to normal mice. Furthermore, following incubation in copper free medium, mutant fibroblasts also retained significantly more copper compared to control. These findings indicate that a Mnk mutation that renders a non-functional transporter affects the ability of the cell to reach copper homeostasis and therefore the cell could not reach copper homeostasis as evidenced by the increased copper accumulation. It is important to point out that control and mutant MNK exhibited perinuclear staining indicative of TGN. However, with increasing copper levels the mutant Mnk in brindled or blotchy cells remained in the TGN, while normal Mnk trafficked to the plasma membrane to efflux copper in order to re-establish copper homeostasis (LaFontaine et al., 1999). Unquestionably, the use of these mice models may serve to provide new insight into the mechanisms by which MNK functions paving the way for impending treatments of this disease.

**Treatment of MD**

There have been few successes in the exploration of a cure for Menkes disease with much of the focus turned to the use of copper-histidine therapy as a potential remedy. Because oral or rectal dispensation of copper is not helpful in treating this disease due to the fact that copper absorption and bioavailability is low along these routes, it is thought that intravenous injection of copper in the form of copper-histidine is a better method to get copper into a patient’s bloodstream (Kodama et al., 1999). It is thought that copper-histidine offers
copper in its most appropriate form because copper uptake by hepatocytes and brain tissue is believed to be physiologically mediated by histidine (Nadal and Baerlocher, 1988). A study conducted by Kreuder et al. (1993) showing promising results with the use of copper-histidine therapy, a 3 month old male patient with MD was given daily intramuscular injections of copper-histidine supplementation and within 6 weeks of therapy, serum and CSF concentrations of copper increased to normal limits. Moreover, analysis of the patient's lumbar CSF showed that dopamine concentrations decreased while norepinephrine concentrations increased to normal limits compared to the levels prior to initiation of therapy. In addition to these important biochemical findings, there was an improvement in the patient's muscular tone, motor activity, alertness, and the improvement of neurological symptoms was observed as evidenced by a decrease in the frequency and duration of this patient's convulsive episodes (Kreuder et al., 1993). It is important to note, however, that such treatment intervention need to take place as early as possible as similar studies conducted in patients at the age of 4, 5, and 7 months did not show similar improvements (Kreuder et al., 1993).

While copper-histidine therapy seems to have some promising results, they can only be described as variable at best as symptom control is evident, but very much limited. It has been hypothesized that, other than genetic heterogeneity and the patient’s age at onset of treatment, the blood-brain barrier may be another explanation for the variable success of copper-histidine therapy as poor clinical outcomes and subnormal brain copper concentration may
continue to be a dilemma despite these therapeutic injections. Thus, studies were developed to determine the effects of intracerebroventricular (ICV) copper-histidine injection in adult rats as a potential strategy to deliver copper to the central nervous system bypassing the blood-brain barrier (Lem et al., 2007). Hence, healthy adult rats were given ICV injection of copper-histidine and their brains imaged using MRI by utilizing copper as an MRI contrast agent due to its paramagnetic properties. Interestingly, the rat brains showed an extensive distribution of copper with 0.5 mcg being the maximum tolerated dose of copper-histidine that did not induce explicit toxicity or reduce lifespan. Furthermore, animals receiving multiple infusions of this dose of copper-histidine did not result in any differences in behavior, activity, or growth compared to saline-injected controls, suggesting that this approach to CNS copper delivery can be a nascent advance in the treatment of MD (Lem et al., 2007). Clearly, studies using ICV copper-histidine injections in the Mo-Br or Mo-BI mouse line may be helpful in guiding the prospect of such a method as much work needs to be done in order to better understand MNK and its role in copper homeostasis. Such efforts are necessary to improve and restore to health to those that suffer from this disease.
CHAPTER III: MATERIALS AND METHODS (Part 1)

Construction of PHMs/DBMCT Expression Vectors. The expression vector encoding PHMs/DBMCT containing a rhodopsin epitope tag in its COOH terminus was generated using the gene splicing by overlap extension (SOE) technique (Horton and Cai, 1990). Two cDNA fragments were amplified by polymerase chain reaction using as templates the pBluescript plasmids carrying the cDNAs for DBM-rhodopsin (rDBM nucleotides 1-2445) (Feng and Angeletti, 1992) and PHMs (rPAM-1 nucleotides 1-1444) (Milgram et al., 1992). Construction of DBM-rhod was described elsewhere (Oyarce et al., 2001). The cDNA fragment encoding rat DBMCT-rhod tag (nucleotides 1549-1866) was spliced onto the cDNA encoding rat PHMs (nucleotides 446-1422) to generate a PHMs/DBMCT-rhod intermediate fragment. The sequence encoding EAENKEKSALMQQLQKYFHIVNRFGE, at the splice junction was verified where the PHMs sequence is shown in bold. The PHMs/DBMCT-rhod intermediate cDNA was inserted into a pCISPHMs expression vector using complementary EcoRV and Hpa I restriction sites to obtain the pCISPHMs/DBMCT-rhod expression vector. Construction of the plasmids encoding DBM-rhod and PHMs was described elsewhere (Milgram et al., 1992; Oyarce and Eipper, 2000).

Construction of DBM-myc expression vectors. For construction of PCINeo DBM-myc, a cDNA fragment encoding DBMCT-myc (rDBM (nucleotides 1574-
was excised from the plasmid PCISP Hispanic DBMCT-myc using Age I and Not I restriction sites. The DBMCT-myc cDNA fragment was then inserted into the PCINeo expression vector carrying the cDNA for DBM (rDBM nucleotides 1-1866) using the Age I and Not I complementary restriction sites.

**Construction of the EGFP/DBMCT expression vector.** A cDNA fragment encoding DBMCT-myc (rDBM (nucleotides 1549-1866)) that contained a BspE I site in its 5' end was constructed by PCR using the PCINeo DBM-myc plasmid as a template and subcloned into pBluescript SK- vector. The BspE I site created as a linker between the EGFP and DBMCT was used to subclone the DBMCT into the pEGFP-C2 vector (BD Biosciences, San Jose, CA). To generate the EGFP-DBMCT expression vector, the DBMCT cDNA fragment was excised from the pBluescript plasmid using BspE I and Sac II and inserted into a pEGFP-C2 expression vector using complementary restriction sites.

**Tissue Culture and Transfections.** AtT-20 cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium (DMEM:F12) containing 10% fetal clone serum (HyClone, Logan, UT) and 10% Nu-Serum (Collaborative Research, Bedford, MA). Stable AtT-20 cell lines were established by co-transfecting the pCISP Hispanic DBMCT-rhod expression vector and the pMT.Neo (Stratagene, La Jolla, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, California). Following transfection, stable cell lines were selected in medium containing G418 (0.5 mg/ml) (Milgram et al., 1992). Cell lines expressing PHMs/DBMCT were
screened by immunofluorescence and Western blot analysis. In addition, PHM activity was determined for the positive clones that were selected for further studies, as described below. For transient transfection of EGFP-DBMCT, WT PC12 cells were pre-incubated in 3 ml of CSFM for 30 min and the EGFP-DBMCT and EGFP control vector were transiently transfected using Lipofectamine 2000. Cells were incubated for 48 h before viewing under an IX50/IX70 Olympus Inverted System Microscope.

**Immunofluorescence Microscopy.** AtT-20 cells expressing the PHMs/DBMCT-rhod chimera were plated onto chamber slides pre-coated with poly L-Lysine (0.1mg/ml) and fixed in ice cold 100% methanol for 15 min and processed as described (Milgram et al., 1992). Following fixation, the slides were washed 3 times in phosphate buffered saline (PBS) and then incubated in PBS containing 2 mg/ml bovine serum albumin (Block) for 20 min. Cells were then incubated with rabbit polyclonal (JH1761) antibody against the PHM domain (rPAM-1 (37-382)) overnight at 4°C (Bell-Parikh et al., 2001. FITC-conjugated goat anti-rabbit IgG (Caltag Laboratories, Burlingame, CA ) was used as a secondary antibody for 1 h in the dark. Both, primary and secondary antisera were diluted 1:1000 in block. Double immunostaining was performed by fixing the cells with 3% paraformaldehyde for 30 min following by permeabilization with 0.075% Triton X-100 for 20 min. Cells were then washed in PBS and incubated in block as described above. Cells were then incubated with the mouse monoclonal Rhodopsin antibody (Hodges et al., 1988), rabbit polyclonal anti-GRP75 (Bip;
Affinity Bioreagents, Golden, CO), and rabbit polyclonal ACTH (1-17) (JH93) (Eipper and Mains, 1980) at room temperature for 2 hours. The anti-sera were diluted 1:1000 (ACTH), 1:6000 (Rhodopsin), 1:9000 (Bip), and 1:10,000 (PHM) in block. FITC-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, Westgrove, PA) were used as secondary antisera. AtT20-PHMs cells were used as control (Milgram et al., 1992; Oyarce and Eipper, 1993; Oyarce and Eipper, 1995; Oyarce and Eipper, 2000). Cells were examined using a Zeiss Axioscope epifluorescence microscope with a photometric PVCAM Nikon Eclipse E800 camera.

**Subcellular Fractionation and Carbonate Wash.** PHMs/DBMTCT cells were homogenized in 0.25 M sucrose, 1 mM MgCl2, 1 mM Na2EDTA, 10 mM Hepes, pH 7.4 using a 4 mm ball-bearing cell homogenizer with a clearance of 0.1555 mm 12 times (H-& Y Enterprise, Redwood City, CA) followed by centrifugation at 800 x g for 5 min to eliminate cellular debris. The homogenate was frozen and thawed 4 times and centrifuged at 356,000 x g for 15 min at 4°C in a Beckman TL100 ultracentrifuge to collect soluble and membrane fractions. To remove peripheral membrane proteins, the membranes were re-suspended in 0.1 M Na2CO3, pH 11.0 and incubated on ice for 30 min. Following the incubation period, samples were centrifuged at 356,000 x g for 15 min to obtain membrane and soluble fractions. Proteins were then fractionated on a 10% SDS-PAGE and analyzed by Western blot. AtT20-PHMs cells were used as control.
Metabolic Labeling and Immunoprecipitation. To elucidate the biosynthesis of the PHMs/DBMCT-rhod chimera, pulse-chase experiments were performed as described previously (Oyarce and Eipper, 2000). Briefly, transfected cells were plated onto poly-L-Lysine (0.1 mg/ml) pre-coated 4 well plates and pre-incubated in methionine free DMEM:F12-air media for 10 min. Cells were then pulse-labeled with 300 μCi [35S] methionine (1 mCi/ml, 1000 Ci/mmol; Amersham) in methionine-free air medium for 20 min. DMEM:F12-air contains sodium HEPES, pH 7.4, rather than NaHCO3. Following the pulse, cells were either extracted immediately (Pulse) or chased in complete DMEM:F12-air for the indicated times (Chase). Following the chase time, cellular proteins were extracted in 20 mM NaTES, 10 mM mannitol, pH 7.0, containing 1% Triton-X-100 (TMT) (Pierce, Rockford, IL) and protease inhibitors (30 μg/ml phenylmethylsulfonyl fluoride, 16 μg/ml benzamidine, 2 μg/ml leupeptin, and 10 μg/ml lima bean trypsin inhibitor). Immunoprecipitations of the media and cell extracts were performed using the PHM antibody by incubation at 4°C for 4 hours or overnight. Immunocomplexes were isolated with protein A-Sepharose by shaking at room temperature for 1 h. Following 3 washes with 50 mM sodium phosphate containing 0.01M Triton X-100 (Pierce, Rockford, IL) (Super E), proteins were fractionated by SDS-PAGE and visualized by fluorography as described (Milgram and Mains, 1993). Pulse-chase experiments using AtT20-PHMs cells were used as control.

Stimulation of Secretion. AtT-20 PHMs/DBMCT-rhod cells cultured on pre-coated poly L-Lysine (0.1 mg/ml) plates were pre-incubated in complete serum
free media (CSFM) containing BSA (0.1 mg/ml) for 3 time periods of 30 min each. Following the pre-incubation periods, cells were incubated in fresh CSFM containing BSA (0.1 mg/ml) for two consecutive 1 h periods to determine basal secretions. To assess stimulated secretion, the cells were incubated in CSFM containing 1 mM BaCl₂ during the subsequent 1 h period. The media collected from the basal and stimulation secretions were centrifuged at 800 x g for 5 min, to eliminate the presence of any cells. Secreted proteins were fractionated on SDS-PAGE and analyzed by Western blot. For quantification of Western blots, non-saturated films were scanned using the BioRad GS-670 Imaging Densitometer and densitometry of the bands were performed using the Molecular Analysis software. Data was expressed using the StatView program and significance was based on p values < 0.05.

**Western Blot Analysis.** Proteins were fractionated on 10% polyacrylamide SDS-PAGE and transferred to Immobilon P polyvinylidene difluoride membrane (Millipore, Billerica, MA) as described previously (Oyarce and Eipper, 2000). The membrane was blocked with milk and then incubated with the PHM or rhodopsin antibody. Proteins were visualized using the ECL kit (Amersham, Piscataway, NJ).

**PHM Activity Assay.** The activity of PHMs/DBMCT-rhod protein was measured using 0.5 μM α-N-acetyl-Tyr-Val-Gly, ¹²⁵I-α-N-acetyl-Tyr-Val-Gly- at pH 5.0. The
reaction is carried out with 0.5 μM CuSO₄, 0.5 mM ascorbate, and 0.18 mg/ml catalase as described previously (Husten and Eipper, 1991).
CHAPTER III: MATERIALS AND METHODS (Part 2)

Tissue Culture and Stable Transfection. Wild-type PC12 cells (WT PC12) were cultured in DMEM:F12 containing 10% fetal clone serum and 10% Nu-Serum. Stable PC12 cell lines were established by transfecting the pCINeoDBM expression vector using Lipofectamine 2000 (Invitrogen, Carlsbad, California). Following transfection, stable cell lines were selected in medium containing G418 (0.5 mg/ml). Cell lines expressing DBM were screened by immunofluorescence and Western blot analysis. Wild-type PC12 cells that respond to depolarization with 100 μM KCl were obtained from Dr. Ronald Holz (University of Michigan, Ann Arbor, MI) and were cultured in DMEM containing 10% fetal clone serum and 5% heat inactivated horse serum (Invitrogen, Carlsbad, CA).

Primary Cultures of Adrenal Chromaffin Cells. Rat adrenal medulla were dissected from adult Sprague Dawley rats, diced with scissors, and rinsed with DMEM/F12-air medium. The diced tissue was then treated with 4 mg/ml collagenase, 1 mg/ml hyaluronidase, 4 μl/ml benzonase, and 10 mg/ml bovine serum albumin, by stirring at 37°C for 20 min. Then, the tissue was diluted in DMEM/F12-air and collected by centrifugation. The adrenal cells were obtained by digesting the tissue with 3 mg/ml trypsin in DMEM/F12-air by stirring for 5 to 10 minutes at 37°C. The cells were then washed in growth medium containing 0.2 mg/ml lima bean trypsin inhibitor, dispersed by pipetting up and down with a flamed pasteur pipette, and passed through a cell strainer. Cells were then
resuspended and plated onto protamine-coated chamber slides and incubated for 2-4 days before use.

**Differential Subcellular Fractionation.** Wild-type PC12 cells, PC12DBM cells and adrenal chromaffin cells prepared as described above were harvested at 4°C in 10 volumes of homogenization buffer containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, and protease inhibitors. Cells were passed through a syringe with a 26 gauge needle six times and then homogenized using a 4 mm ball bearing homogenizer with a clearance 0.1555 mm 12 times (H&Y Enterprises, Redwood City, CA) as described (El Meskini and Galano, 2001). The Beckman TL Optimum Ultracentrifuge using the TL 100.2 rotor was utilized for the differential subcellular fractionation. Cell debris was removed by centrifugation at 800 x g for 5 min giving a pellet (P1). The supernatant (S1) was then centrifuged at 4,000 x g for 15 min to obtain a second pellet (P2) and soluble fraction (S2). A third pellet, P3, was also collected by centrifugation of S2 at 10,000 x g for 15 min. The supernatant S3 was centrifuged at 350,000 x g to generate a P4 pellet and a cytosolic fraction. Pellets were resuspended in TMT and aliquots equivalent 10% of sample were fractionated on SDS-PAGE and visualized by Western blot.

**Sucrose Density Gradient Fractionation.** The P2 and P3 pellets, which contain secretory granules, in addition to other subcellular organelles, were resuspended in 0.32 M sucrose and further fractionated on a discontinuous sucrose gradient, as described (El Meskini and Galano, 2001). The density
gradient for the P2 pellet consisted of 150 μl of 0.4 M sucrose, 200 μl of 0.6 M, 0.8 M, and 1.6 M sucrose, and 325 μl of 1.0 M, 1.2 M, and 1.4 M sucrose. The P3 pellet was separated on a sucrose gradient consisting of 150 μl of 0.6 M sucrose, 200 μl of 0.8 M and 1.0M sucrose, 350 μl of 1.1 M, 1.2 M and 1.4 M sucrose, 300 μl of 1.6 M sucrose, and 250 μl of 2.2 M sucrose. The gradients were centrifuged at 120,000 x g for 2 hours using the TL55 swinging bucket rotor in a Beckman TL Optimum Ultracentrifuge. Individual fractions were collected by taking 157 μl from the top of the gradient and aliquots containing an equal volume of each sample were analyzed via Western blot using the antibody to the carboxy-terminus of MNK (CT 77) (Steveson et al., 2003), DBM (Oyarce and Eipper, 2000), CGA, synaptophysin (p38), synaptotagmin (p65), and syntaxin-6 (syn6) (BD Biosciences, San Jose, CA).

**Immunofluorescence Microscopy.** PC12 cells plated on chamber slides pre-coated with poly L-lysine were fixed with ice cold methanol for 15 min and incubated with antibodies to MNK and the secretory granule markers, DBM and CGA as described (Oyarce and Eipper, 2000; Oyarce et al., 2001; Steveson et al., 2003) A FITC-conjugated secondary antibody was used and the cells were examined using the Leica TCS SP2 inverted confocal microscope.

**Bathocuproine disulfonate and Copper chloride treatment.** Cells plated on either precoated chamber slides or 60 mm dishes were treated with 50 μM bathocuproine disulfonate (BCS) for 16 hours at 37°C for copper chelation.
studies. Cells treated with copper chloride were incubated with 200 μM CuCl₂ for 5 hours at 37°C. For copper treatment dose response experiments, cells were plated onto poly L-lysine coated 12 well plates and were treated with 0, 25, 50, 100, 200, 400, and 500 μM copper chloride for 5 h. For time course experiments, cells were treated with 200 μM copper chloride for 5 min, 15 min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h, and 5 h at 37°C. Control cells were incubated in medium without CuCl₂ or BCS.

**Biotinylation of Cells treated with Copper.** Following copper treatment, cells were rinsed with 1 ml ice cold PBS containing 0.5 mM MgCl₂ and 1 mM CaCl₂ (wash buffer) on ice for 5 min prior to biotinylation. The cells were then incubated with 3 mg/ml EZ-link Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) in wash buffer for 30 min on ice. Following this incubation, cells were rinsed twice with wash buffer for 5 minutes and the excess biotin was quenched with 100 mM Glycine, pH 7.4, for 10 min at 4°C. The cells were then washed three times in wash buffer and proteins were extracted in 250 μl TMT containing protease inhibitors. Extracts were frozen and thawed three times, centrifuged at 10,000 rpm for 5 min in an eppendorf centrifuge, and transferred to a new tube. An aliquot of the cell extract containing 60 μg of protein was diluted in 1ml of Super E and the biotinylated proteins were isolated by incubation with 250 μl of Immobilized Neutravidin agarose beads (Pierce, Rockford, IL) in a rotor at room temperature for 45 min. Following the incubation period, the biotin:beads complex was then collected by centrifugation at 10,000 rpm for 1 min. After
washing the beads with Super E twice and once with 50 mM sodium phosphate buffer, the proteins were eluted by incubation in SDS-PAGE sample buffer at 50°C x 5 min. Proteins were fractionated by SDS-PAGE and biotinylated MNK was analyzed by Western blot.

**Stimulation of PC12 cells.** WT PC12 cells plated onto poly L-lysine precoated slides, and 4 or 12 well plates were rinsed in PBS for 5 min at 37°C. After the rinse, cells were incubated with 10 mM KCl in PBS for 0, 5, 10, and 20 min at 37°C to stimulate secretion. Control cells were incubated in PBS without KCl. Following stimulation, cells were either biotinylated or were fixed in methanol for immunostaining as described above.

**Quantification and Statistical Analysis.** For quantification of Western blots, non-saturated films were scanned using the BioRad GS-670 Imaging Densitometer and densitometry of the bands were performed using the Molecular Analysis software. Data was expressed using the StatView program and significance was based on p values < 0.05.
FULLY ACTIVE PHMs/DBMCT CHIMERA IS EXPRESSED IN AT-T-20 CELLS. To elucidate the role of the COOH-terminal domain of DBM in sorting and trafficking of DBM, we created the PHMs/DBMCT chimera by attaching the DBM COOH-terminal (DBMCT) containing a rhodopsin tag to the reporter PHMs protein (Fig. 7). The chimera was expressed in AtT-20 cells, a neuroendocrine cell that

**Figure 7. Construction of the PHMs/DBMCT chimera**

Figure 7. Scale representations of DBM rhod chimera (Feng and Angeletti, 1992), full length PHMs and the PHMs/DBMCT chimera are depicted. DBM representation illustrates residues that participate in disulfide linkages and N-glycosylation sites (red lollipop sticks). Predicted molecular masses (kDa) and the site of the rhodopsin tag (Rhod, purple) are shown.
contains secretory granules, and the role of the DBMCT was investigated by evaluating its effect on the behavior of the reporter PHMs protein. AtT-20 cells expressing the soluble PHMs protein were examined for comparison. Aliquots of cell extract from AtT20 PHMs/DBMCT and AtT20 PHMs containing equal amount of PHM activity were fractionated by SDS-PAGE and subjected to Western blot analysis using a PHMs antibody. As shown in Fig. 8, PHMs/DBMCT was expressed as two molecular species with apparent molecular masses of 51 kDa and 48 kDa. The fact that both species are substantially greater than the

Figure 8. Analysis of PHMs/DBMCT by Western blot

Figure 8. Cell extracts were prepared from AtT-20 PHMs/DBMCT, AtT-20 PHMs, and non-transfected (NT) AtT-20 cells. Aliquots containing 70 pmol/hour PHM activity were fractionated by SDS-PAGE and PHM was identified by Western blot using the PHM antibody. Similar results were obtained in three independent experiments.
the molecular mass of PHMs (38 kDa) indicates the presence of the COOH-terminal domain of DBM in the chimera. As expected, no proteins of the size of PHMs/DBMCT or PHMs were detected in non-transfected AtT-20 cells (NT). Most importantly, similar amounts of PHMs/DBMCT protein and PHM protein

Figure 9. Subcellular Fractionation of PHMs/DBMCT and PHMs

Figure 9. Secreted (Sec), soluble (Sol), and membrane (Mb) fractions were prepared from AtT-20 cells expressing PHMs/DBMCT as well as from AtT-20 PHMs. Proteins were fractionated by SDS-PAGE and analyzed by Western blot using the PHM antibody. These results were obtained by three independent experiments.
yielded 70 pmol/h of PHM activity indicating that the chimera folds properly and it is as active as the control PHMs protein.

**Subcellular Distribution of PHMs/DBMCT.** To further investigate the properties of the PHMs/DBMCT chimera, subcellular fractionation was carried out. Stably transfected AtT-20 cells expressing PHMs/DBMCT or PHMs were separated into soluble and membrane fractions and analyzed by Western blot with the PHM antibody. As shown in Fig. 9, both molecular species of PHMs/DBMCT were found in similar amounts in the soluble and membrane fractions. In contrast, PHMs protein was recovered primarily in the soluble fraction with no significant amount of PHMs in the membrane fraction. Variable amounts of monofunctional soluble PHMs has been observed to be partially associated with membranes in secretory granules from rat hypothalamus and hippocampus (Oyarce and Eipper, 1995) as well as in AtT-20 PHMs cells (Oyarce et al., 2001). In addition, the 51 kDa and 48 kDa PHMs/DBMCT forms as well as 38 kDa PHMs were secreted into the culture medium.

The association of the PHMs/DBMCT chimera with membranes was further investigated by incubating membranes with sodium carbonate, pH 11.0, for 30 min on ice to dissociate peripheral proteins (Fig. 10) (Oyarce et al., 2001). For comparison, membranes not treated with sodium carbonate were loaded on the gel (Fig. 10, Mb). After carbonate treatment, a significant amount of both forms of PHMs/DBMCT remained associated with the membrane (Fig. 10, CwMb). In contrast, all the PHMs protein present in the membrane was removed.
by the carbonate treatment (Fig. 10, Cw) as expected for a soluble protein.

These data indicate that the COOH-terminal domain of DBM when attached to PHMs confers properties to the chimera that allow its partial membrane association.

**Figure 10. Carbonate Wash of membranes containing PHMs/DBMCT and PHMs**

The membrane fractions were washed in 0.1 M Na₂CO₃, pH 11.0 (Carbonate wash) as described in Methods. Membranes before the wash (Mb), membranes after the wash (CwMb), and the carbonate wash (Cw) are depicted for AtT-20 cells expressing PHMs/DBMCT and AtT-20 PHMs. Proteins were fractionated by SDS-PAGE and analyzed by Western blot using the PHM antibody. Molecular masses are shown as kDa. Similar results were obtained from three independent experiments.
PHMs/DBMCT is Slowly Secreted from AtT-20 Cells. To study the biosynthesis and secretion of PHMs/DBMCT expressed in AtT-20 cells, metabolic labeling experiments were carried out. The biosynthesis and secretion of PHMs in AtT-20 cells were analyzed for comparison. PHMs/DBMCT and PHMs were immunoprecipitated from cell extracts (E) and culture medium (M),

**Figure 11. Biosynthesis and secretion of PHMs/DBMCT and PHMs**

![Graph showing biosynthesis and secretion of PHMs/DBMCT and PHMs](image)

Figure 11. AtT-20 cells expressing PHMS/DBMCT were labeled with [³⁵S]-methionine for 20 min (Pulse, P) and chased in CSFM-air for 30 min, 1h, 2h, 3h, 4h, and 6h. PHMs/DBMCT was immunoprecipitated from cell extracts (E) and culture media (M) using the PHM antibody, analyzed by SDS-PAGE and detected by fluorography. The inset shows a longer exposure time for the detection of secreted PHMs/DBMCT. AtT-20 cells expressing PHMs were pulse labeled with [³⁵S]-methionine and chased in the same manner as that of PHMs/DBMCT described above. The molecular masses are indicated in kDa. Similar results were obtained by three independent experiments.
fractionated by SDS-PAGE, and detected by fluorography. After the pulse incubation (Fig. 11, P), PHMs/DBMCT was detected as single specie of 51 kDa that was identical in size to the larger form of the chimera identified by Western blot (Fig. 8). Between 30 min and 3-h of chase, all the newly synthesized PHMs/DBMCT that was recovered maintained its 51 kDa molecular mass, indicating that the chimera is still intact. Interestingly, significant amount of the small 48 kDa form was observed only after 4-h chase suggesting that PHMs/DBMCT protein undergoes proteolytic processing in AtT-20 cells. The PHMs protein was recovered as a 40 kDa protein after the pulse and it matured into a 38 kDa protein due to the removal of its pro-region as shown previously (Milgram et al., 1992) (Fig. 11). Processing of PHMs was completed between 30 min and 2-h chase in agreement with previous studies (Oyarce et al., 2001).

Spent medium (M) was analyzed to evaluate the secretion of newly synthesized PHMs/DBMCT protein. Secretion of the 51 kDa form from AtT-20 cells was detected at 3-h of chase, while there was a lag time of 4-h before the 48 kDa form was readily detected in the culture medium (Fig. 11, M). A prolonged chase time of 16 h clearly showed secretion of both 51 kDa and 48 kDa forms of the chimera (Fig. 11, inset). A significant amount of both PHMs/DBMCT forms remained in the cell after 6-h of chase. In contrast, secretion of the 38 kDa PHMs protein from AtT-20 cells was observed at 1-h of chase (Fig. 11, bottom panel). An increased in the amount of PHMs secreted into the culture medium was observed with time, while a small amount of PHMs remained in the cell after 6-h chase. This data indicates that the attachment of
the COOH-terminal domain of DBM to the PHMs protein reduces the rate of
PHMs secretion.

**COOH-terminal Domain of DBM is Proteolytically Processed in AtT-20**

**Cells.** To further investigate whether the smaller 48 kDa form is originated by
proteolytic
processing of the COOH-terminal domain of PHMs/DBMCT, we took advantage
of the presence of a rhodopsin tag on the COOH-terminus of the chimera as
described above (Fig.7). AtT-20 cells expressing PHMs/DBMCT were extracted,
fractionated by SDS-PAGE and the chimera was analyzed by Western blot using
both the PHM and rhodopsin antisera. As shown in Figure 12, the PHM antibody
detected both the 51 kDa and 48 kDa forms of PHMs/DBMCT, while the
rhodopsin antibody only detected the 51 kDa form. The inability of the rhodopsin
antibody to detect the 48 kDa form indicates that a cleavage in the COOH-
terminal domain of DBM has removed the rhodopsin tag from the PHMs/DBMCT
protein. Most importantly, these results indicate that the smaller 48 kDa form is
originated from the 51 kDa form by proteolytic processing of the DBM COOH-
terminal domain in the chimera. It should be noted that a cleavage of only the
rhodopsin tag from the DBM COOH-terminal domain of PHMs/DBMCT only
removes 9 amino acids, yet this does not reflect the size difference between the
PHMs/DBMCT forms (Fig. 12B, rhod, red letters). Therefore, we estimated that
the cleaved fragment is approximately 25 to 30 amino acids in length.
Figure 12. Western blot analysis of PHMs/DBMCT processing

A. Western blot

PHM Ab  Rhod Ab

51 kDa  48 kDa

B. PHMs/DBMCT- Rhod Sequence

Met AGRA SGLL LLLL LALQLSS CLAFRSPLSVFKRFKETTRSFSENCGLTP
VTPL DASDFALDIR Met PGVTPKESDTYFC Met SLRPVDHEEAFVIDFKFRAS
Met DTVHH Met LFGCN Met PSSGYSWFCDGTCXDKANILAYAWNAPPTFLP
KGYGFRRGGETGSKYFVQLQYHGDISAPRDNHKDCSGYSVHLTRVPQPLIAG
Met YL Met SVTVHPPGKEKVNNADISCQYK Met YP Met HFAYRVTHHLGKV
VSYRVNNGQWTLIGRQPLOPAFYPEHPVDVFDTDILAARCVFTGEGRTE
ATHI GCAKLSDE Met CNLYY Met YY Met EAKYALSF Met TCTKNVAPD Met FRTIPAE
ANIP PVKD Met V Met HHKEAEKESAL Met QQLQKYPhVNRFGNEECVT
TCPQAASVPQFGASVFWNSNFNRMet LKALVNYAPISVHCNKTSAVRFPGNWNILQ
PLP NITSAVEEPDP RCPQRQTRG PAHPVFVITHGGRH CATE T S V A P A

[Diagram showing the sequence with PHMs in black, DBM in blue, and rhodopsin tag in red with a putative cleavage site.]

Figure 12.  A.)  Cell extracts of AtT-20 cells expressing PHMs/DBMCT were prepared and fractionated by SDS-PAGE. Detection of the proteins were done by Western blot analysis with the PHM antibody and the rhodopsin antibody. Molecular masses are shown in kDa. The 48 kDa band is not detected with the rhodopsin antibody. B.) The sequence of PHMs/DBMCT is shown where the PHMs portion of the chimeric protein is indicated in black and the C-terminal of DBM is shown in blue. The rhodopsin tag (red letters) is shown attached to the C-terminal of DBM by a linker region containing the amino acids GA (gray letters). Based on the difference in size of the chimera forms, the potential cleavage site in the C-terminal is indicated. Similar results were obtained by three independent experiments.
Based on the PHM/DBMCT amino acid sequence, a putative cleavage site has been identified in the COOH-terminal domain of DBM (Fig. 12B, underlined letters).

**Subcellular localization of PHMs/DBMCT in AtT-20 cells.** To analyze the subcellular localization of PHMs/DBMCT, AtT-20 PHMsDBMCT cells were subjected to immunofluorescence microscopy analysis using the PHM antibody. As shown in Fig. 13, a diffuse reticular staining for PHMs/DBMCT was observed throughout the cell, however, staining was excluded from the nucleus (Fig. 13, arrow). The diffuse staining resembles the staining of Bip, an ER protein, suggesting that a significant amount of the chimera is present in the ER of AtT-20 cells. In addition to the ER staining, PHMs/DBMCT also was localized at the tips of the cell where secretory granules are located (Fig. 13, SG, arrowheads). Control experiments using AtT-20 PHMs cells show that the PHMs protein is localized to secretory granules (Fig. 13, SG, arrowheads) as well as the perinuclear TGN region (Fig. 13, arrow) as previously shown (Milgram et al., 1992). A comparison of PHMs/DBMCT localization was also done with immunostaining of AtT-20 cells with antibody to β-endorphin, a cleavage product of pro-opiomelanocortin (POMC), which is stored in secretory granules (Eipper et al., 1980; Zhou and Bloomquist, 1993). Immunostaining with this antibody illustrates secretory granule localization at the tips of cell processes, which resembles the localization of PHMs/DBMCT in these structures (Fig. 14). The subcellular compartments containing PHMs/DBMCT in AtT-20 cells were further
Figure 13. AtT-20 cells transfected with PHMs/DBMCT were visualized by immunofluorescence using the PHM antibody. AtT-20 PHMs cells were analyzed for comparison. The nucleus is indicated with an arrow. Secretory granules (SG) are depicted with arrowheads at the tips of cellular processes where they are concentrated in AtT-20 cells. The TGN is also shown in AtT-20 cells expressing PHMs with a bold arrow. Similar results were obtained by three independent experiments.

analyzed by comparing the distribution of the chimera to organelle marker proteins by double immunostaining. AtT-20 cells synthesize POMC that is a precursor to neuropeptides such as ACTH, β-endorphin and α-melanotropin (Eipper and Mains, 1980). Because the POMC products are stored in secretory granules in AtT-20 cells, the ACTH peptide was used as an organelle marker for secretory granules (Eipper and Mains, 1980; Zhou and Bloomquist, 1993). PHMs/DBMCT visualized with the rhodopsin antibody showed a diffuse staining
Figure 14. Localization of PHMs/DBMCT and β-endorphin in AtT-20 cells

Figure 14. AtT20 cells transfected with PHMs/DBMCT was visualized by immunofluorescence using the PHM antibody. AtT-20 PHMs/DBMCT cells stained with β-endorphin antibody was used to identify the localization of secretory granules. The nucleus is indicated with an arrow. Secretory granules (SG) are depicted with arrowheads at the tips of cellular processes where they are concentrated in AtT-20 cells. Similar results were obtained in three independent experiments.

throughout the cytoplasm in addition to staining at the tips of the cell (Fig. 15A).
The PHMs/DBMCT staining with the rhodopsin antibody is in agreement with the staining observed when using the PHM antibody (Fig. 13). AtT-20 PHMs/DBMCT cells immunostained with the ACTH antibody showed peptide staining at the tips of the cells where secretory granules (SG) are located, confirming its granule localization (Fig. 15B, arrowhead). AtT-20 PHMs/DBMCT cells visualized simultaneously with the monoclonal rhodopsin antibody and the
Figure 15. Subcellular localization of PHMs/DBMCT and organelle markers in AtT-20 cells.

Figure 15. To compare the localization of PHMs/DBMCT (A) with that of the secretory granule marker ACTH (B) and the ER protein Bip (E), cells were fixed and stained simultaneously with the monoclonal antibody to rhodopsin and rabbit anti-ACTH or anti-Bip. The merged images showed co-localization of PHMs/DBMCT and ACTH in secretory granules (C, SG, arrowheads) at the tip of the cellular processes as well as co-localization with Bip in the ER (F, ER, bold arrow). Similar results were obtained in three independent experiments.

A polyclonal ACTH antibody showed co-localization of both proteins to secretory granules (SG), as shown in yellow (Fig. 15C, arrowhead, merge). Cells stained with the ER-specific Bip antibody showed diffuse staining typical of ER (Fig. 15E, block arrow) (Cortes and Winograd, 2003). Double immunostaining analysis
using the rhodopsin and Bip antisera confirmed the co-localization of
PHMs/DBMCT and Bip to the ER (Fig. 15F, merge). These results indicate that
PHMs/DBMCT is localized to secretory granules as well as to the ER in AtT-20
cells. The PHMs/DBMCT staining pattern was similar to that of soluble PHMs in
its SG localization, but it differs in its ER localization (Figs. 9 and 15).

**PHMs/DBMCT Can Be Stimulated to Secrete from AtT-20 Cells.** To further
confirm that PHMs/DBMCT is stored in secretory granules, we determined
whether the chimera reached a stimulatable secretory compartment in AtT-20
cells. Secretion of PHMs/DBMCT under basal conditions was determined over
two 1-h periods, before addition of the secretagogue BaCl₂ for a final 1-h
collection period. Proteins were analyzed by Western blot as described (Oyarce
and Eipper, 2000). As shown in Fig. 16A, both forms of PHMs/DBMCT have
similar basal secretion rates and the addition of BaCl₂ stimulated the secretion of
both forms of the chimera. Furthermore, quantitation of PHMs/DBMCT secretion
showed that the addition of secretagogue caused a 1.6-fold increase in secretion
of the 51 kDa form (88 +/- 16.9 ARB (P < 0.0004), while the secretion of the 48
kDa form showed a larger increase of 2.3-fold (149 +/- 5.9 ARB, P< 0.0001) (Fig.
16B). These data confirmed that both forms of the PHMs/DBMCT chimera were
stored in secretory granules in AtT-20 cells and that they differ in their abilities to
undergo stimulated secretion.
Figure 16. Stimulation of PHMs/DBMCT secretion from AtT-20 cells.

A.

Figure 16. AtT-20 PHMs/DBMCT cells were incubated in CSFM containing BSA for two sequential one hour periods (Basal1 and Basal2), and then stimulated with 1 mM BaCl₂ (Stimulation) for 1 h to assess stimulated secretion. A.) Western blot analysis of secreted PHMs/DBMCT was done using the PHM antibody. The molecular masses are indicated in kDa. B.) Quantification of relative amount of basal and stimulated secretion of the two forms of PHMs/DBMCT in AtT-20 cells was determined using the Molecular Analysis software (BioRad, California) where asterisks indicate statically significant differences between stimulated and basal secretion with P < 0.0004 (*) and P < 0.0001 (**). These results are representative of seven independent experiments.

Full length DBM may undergo an endoproteolytic cleavage in PC12 cells.

We attempted to determine whether the endoproteolytic processing that was
found in PHMs/DBMCT was due to the folding of the DBMCT in the chimeric protein or if this cleavage also occurred in the full length DBM. Since we wanted to determine whether or not the proteolytic processing of DBM was cell type specific, a construct of the full length DBM with a myc tag in its COOH terminus was generated and expressed in PC12 cells. PC12 cells are a tumor cell line that derives from the adrenal medulla that synthesize catecholamines, and contain regulated secretory pathway. DBM is sorted to secretory granules in PC12 cells while it does not traffic to secretory granules in AtT-20 cells due to cell-type specific trafficking and sorting (Oyarce and Eipper, 2000). Western blot analysis of PC12 cells transfected with DBM-myc showed that full length DBM can be detected with the DBM antibody (Fig. 17). While the myc antibody was able to detect the myc control protein, Rab-myc, it did not detect full length DBM-

Figure 17. Western blot Analysis of PC12 DBM-myc

![Western blot analysis of PC12 DBM-myc](image)

Figure 17. Western blot analysis of PC12 DBM-myc was done with the DBM antibody and Myc antibody. Rab-myc was used as a control for the detection with the Myc antibody.
myc. Thus, the inability to detect the myc tag in PC12 DBM-myc suggests that full length DBM-myc may undergo endoproteolytic cleavage in PC12 cells.

**EGFP/DBMCT immunostaining suggests localization to the endoplasmic reticulum.** It has been shown that PHMs when expressed by itself in AtT-20 cells was localized to secretory granules (Milgram et al., 1992). Therefore, it is difficult to evaluate the sorting and trafficking information in DBMCT when it is attached to the PHMs protein. In an attempt to determine whether the DBM COOH-terminus has trafficking information to secretory granules or if the recruitment of the chimeric protein was due to PHMs intrinsic sorting information, a construct was generated containing DBM COOH-terminus appended to EGFP. This EGFP/DBMCT chimera was transiently expressed in PC12 cells and live cells were analyzed by immunofluorescence microscopy. In control cells, EGFP showed a diffuse, non-specific staining throughout the cell, including the nucleus (Fig. 18). In comparison to the EGFP control, however, EGFP/DBMCT showed a diffuse cytoplasmic staining pattern resembling endoplasmic reticulum staining. More importantly, there was not clear vesicular staining indicating the presence of EGFP/DBMCT in secretory granules in PC12 cells. This staining pattern suggests that either EGFP/DBMCT protein is not folding properly and is trapped within the endoplasmic reticulum, and/or that the DBMCT domain does not contain trafficking information for the sorting of the EGFP/DBMCT chimera to secretory granules. If DBMCT does not contain trafficking information, it may only provide structural information for dimerization, essential for DBM function.
Figure 18. Wild-type PC12 cells were transiently transfected with the EGFP (A-C) and EGFP-DBMCT (D-F) expression vector using Lipofectamine 2000. Phase contrast micrographs of PC12 cells expressing EGFP control and EGFP-DBMCT are shown in panels A and D, respectively. Immunostaining of EGFP in PC12 cells is shown in panel B, while panel E shows EGFP-DBMCT staining. A merge of the phase contrast micrographs and the EGFP (B) and EGFP-DBMCT (F) staining is also shown. Immunostaining within the ER is indicated by the arrowhead. The nucleus is shown with an arrow in all panels.
CHAPTER IV: RESULTS (Part 2)

Subcellular Localization of MNK in Adrenal Chromaffin and PC12 Cells.

Previous studies using transfected fibroblasts, have shown that the Menkes copper transporter is localized to the TGN (Ackland, 1997; Ackland et al., 1997; Ackland et al., 1999; Dierick et al., 1997; Francis et al., 1998; LaFontaine et al., 1998a; LaFontaine et al., 1998b). Since, little is known about MNK in endocrine cells we hypothesize that MNK has a differential subcellular distribution in these cells that contain regulated secretory pathway and resident cuproenzymes. In order to answer the question of subcellular localization of MNK in endocrine cell types, immunofluorescence microscopy was carried out using adrenal chromaffin cells and its tumor cell line, PC12 cells. The reason why PC12 cells were used for these experiments is that if the subcellular localization of MNK is the same between the two cell types, then further characterization of MNK can be continued in PC12 cells due to the ease in which they can be manipulated and maintained in the laboratory. For an initial assessment of MNK localization, adrenal chromaffin and PC12 cells were subjected to immunofluorescence microscopy using the MNK antibody. As seen in Fig. 19, staining of MNK in both adrenal chromaffin and PC12 cells exhibits a punctate, vesicular staining pattern throughout the cytosol that resembles the staining of secretory granules resident proteins (SG, arrowheads). Staining in the nucleus of both cell types is not observed. Interestingly, this finding differs from the perinuclear staining of MNK in fibroblasts, suggesting its presence in secretory granules in endocrine cells.
Figure 19. Immunostaining of Adrenal Chromaffin and PC12DBM Cells

Figures 19. Adrenal chromaffin and PC12DBM cells were fixed in ice-cold methanol and stained with the MNK antibody. The arrowheads identify punctate vesicular staining previously identified as secretory granules (SG), while the nucleus is shown by the long arrow.

Figure 20. Schematic representation of differential centrifugation

Figure 20. Adrenal tissue was homogenized and centrifuged to obtain a P1 pellet and S1 supernatant fraction. Then, supernatant fractions (S1, S2, and S3) were further centrifuged to obtain the subsequent pellet fractions P2, P3, and P4. S4 is the cytosolic fraction.
In order to further explore the localization of MNK, subcellular fractionation by differential centrifugation was performed to obtain pellet fractions that are enriched in different subcellular organelles (Fig. 20). Western blot analysis of the pellet fractions was performed to identify where MNK and organelle markers were found in the respective pellets. Following fractionation, the adrenal tissue and PC12 cells were separated into a homogenate fraction and pellet fractions.

**Figure 21. Western blot analysis of pellet fractions from adrenal chromaffin cells**

![Western blot analysis](image)

Figure 21. Pellet fractions obtained by differential centrifugation were resuspended in TMT and 10% aliquots were fractionated by SDS-PAGE. Pellets and cytosolic fractions were analyzed by Western blot using the MNK antibody and antibodies against the secretory granule markers DBM, CGA and p65 as well as p38 (synaptic like microvesicles) and Syn6 (TGN).
P1, P2, P3, and P4 pellets and a cytosolic fraction. Western blot analysis of pellet fractions derived from adrenal cells showed that MNK is enriched in P2 and P3 pellets with the P3 fraction containing more MNK protein (Fig. 21). Because the MNK staining pattern suggested the presence of MNK in secretory granules, we analyzed the distribution of organelle markers in the pellet fractions containing MNK. Our results demonstrate that the secretory granule proteins DBM, CGA and p65 were also enriched in the P2 and P3 pellets (Fig. 21). Synaptotagmin (p65) is found in secretory granules and synaptic microvesicles. The synaptic microvesicle marker p38 was enriched in P3 and P4, while Syn6, a TGN and synaptic like microvesicle marker was also enriched in P3. Interestingly, a similar distribution of MNK and organelle markers was also observed in the adrenal tumor cell line, PC12 cells (Fig. 22). In PC12 cells, MNK was once again found to be enriched in the P2 and P3 fractions together with the secretory granule proteins DBM, CGA, p65, and Cythb561. Overall, our results indicate that secretory granules and TGN are heavily concentrated in P2 and P3 pellet fractions following differential centrifugation. Since the distribution pattern of the marker proteins is consistent with the MNK pattern, these findings suggest that the MNK protein may be localized to secretory granules as well as TGN in adrenal chromaffin and PC12 cells.

Sucrose density gradient fractionations suggest that MNK is found in secretory granules and TGN. To better characterize the subcellular localization of MNK, the pellets enriched in MNK obtained from differential centrifugation
Figure 22. Western blot analysis of pellet fractions from PC12 DBM cells

Figure 22. Pellet fractions obtained via differential centrifugation were resuspended in TMT and 10% aliquots of the pellets and cytosolic fraction were fractionated by SDS-PAGE. Western blot analysis was done to compare the distribution of MNK to that of secretory granule markers DBM, CGA, p65, and Cytb561 as well as p38 (synaptic like microvesicles) and Syn6 (TGN).

were further subjected to sucrose density gradient fractionation. An aliquot of the P2 pellet from adrenal chromaffin cells was further analyzed on a sucrose density gradient containing layers of sucrose from 0.3 to 1.6 M, designed to allow heavier organelles to collect at the bottom of the gradient. In the P2 gradient (Fig. 23), MNK showed a bimodal distribution, separated into a lighter region containing fractions 6-9 and a heavier region corresponding to fractions 12-14. Since our initial characterization of MNK indicates that MNK may be localized to secretory
Figure 23. Adrenal chromaffin cell P2 sucrose density gradient fractionation

Figure 23. P2 pellet obtained by differential centrifugation was resuspended in 0.32 M sucrose and fractionated on a sucrose density step gradient containing 0.3 M to 1.6 M sucrose. Fractions of 157 μl were taken from top to bottom of the gradient. Equal aliquots of each fraction were further fractionated on SDS-PAGE and Western blot analysis for the distribution of MNK and organelle markers was carried out using antisera against secretory granule markers DBM, CGA, and p65, TGN (Syn6), and synaptic like microvesicles (p38).

granules and TGN, MNK distribution was then compared to Syn 6 and secretory granule markers DBM, CGA, and p65. The secretory granule proteins DBM, CGA, and p65 were found in the denser regions of the gradient with an enrichment in fractions 13 and 14 which contain significant amounts of MNK.
The TGN marker, Syn 6, was enriched in fractions 5-8 and co-distributed with MNK in the lighter fractions of the gradient. p38 was enriched primarily in

Figure 24. Adrenal chromaffin cell P3 sucrose density gradient fractionation

Figure 24. P3 pellet obtained by differential centrifugation was resuspended in 0.32M sucrose and fractionated on a sucrose density step gradient containing 0.3M to 2.2M sucrose. Fractions of 157 µl were taken from top to bottom of the gradient. Equal aliquots of each fraction were further fractionated on SDS-PAGE and Western blot analysis for the distribution of MNK and organelle markers was carried out using antisera against secretory granule markers DBM, CGA, and p65, TGN (Syn6), and synaptic like microvesicles (p38).
fractions 8-11 and did not show significant co-localization with MNK. Analysis of
the P3 pellet on sucrose density gradient showed that MNK was distributed in the
lighter fractions 4-7 as well as in the heavier fractions 8-11 (Fig. 24). The
localization of the secretory granule markers DBM, CGA, and p65 in the heavier
fractions indicates the presence of secretory granules in these fractions. The
distribution pattern of p38 demonstrates that synaptic like microvesicles are
present primarily in the lighter fractions 4-7. Furthermore, Syn6 in the lighter
fractions indicate enrichment of TGN in fractions 4-8. Since the P3 gradient was
designed to avoid pelleting secretory granules, the presence of DBM, CGA, and
p65 in fractions 12-14 indicates that the P3 gradient was a bit overloaded.
However, the different distribution of Syn6 and secretory granules markers in the
gradient as well as the co-distribution of these markers with MNK clearly indicate
that MNK is localized to the TGN and secretory granules in adrenal chromaffin
cells. Because we are interested in using the PC12 cells as a model for the
adrenal chromaffin cells, fractionation studies were performed to determine
whether the subcellular distribution of MNK in PC12 cells was similar to that in
the adrenal cells. As shown in Fig. 25, MNK had a bimodal distribution with an
enrichment in fractions 9-11 and in the heavier fractions 12-14 in the P2
gradient. Furthermore, MNK in the heavier fractions co-distributed with the
secretory granule markers DBM and CGA, while MNK shared a similar
distribution with Syn6, a TGN marker, in lighter fractions 8-11. In the P3 sucrose
density gradient (Fig. 26), MNK was localized to lighter fractions 4-8 that also
contained Syn6 (Figure 26) as well as to fractions 9-11 containing secretory
Figure 25. P2 pellet obtained by differential centrifugation was resuspended in 0.32 M sucrose and fractionated on a sucrose density step gradient containing 0.3 M to 1.6 M sucrose. Fractions of 157 ml were taken from top to bottom of the gradient. Equal aliquots of each fraction were further fractionated on SDS-PAGE and Western blot analysis for the distribution of MNK and organelle markers was carried out using antisera against secretory granule markers DBM, CGA, and p65, TGN (Syn6), and synaptic like microvesicles (p38).

Just as observed in adrenal chromaffin cells, the localization of p38 to lighter fractions 5-7 indicates the presence of synaptic like microvesicles in these fractions. Thus, these results indicate that in PC12 cells MNK also is localized to the TGN and secretory granules consistent with the findings in adrenal chromaffin cells. Because of these results, we used the PC12 cell
Figure 26. PC12 DBM P3 sucrose density gradient fractionation

Figure 26. P3 pellet obtained by differential centrifugation was resuspended in 0.32 M sucrose and fractionated on a sucrose density step gradient containing 0.3 M to 2.2 M sucrose. Fractions of 157 μl were taken from top to bottom of the gradient. Equal aliquots of each fraction were further fractionated on SDS-PAGE and Western blot analysis for the distribution of MNK and organelle markers was carried out using antisera against secretory granule markers DBM, CGA, and p65, TGN (Syn6), and synaptic like microvesicles (p38).

system for further studies instead of the primary adrenal chromaffin cells from rat adrenal glands.

Re-localization of MNK to the plasma membrane following depolarization of PC12 cells with KCl. In an attempt to further analyze the subcellular localization of MNK in PC12 and adrenal chromaffin cells, MNK immunostaining was compared to the staining of secretory granule markers in these cells. As shown
Figure 27. Chromaffin cells (top panel) and PC12 cells (bottom panel) were plated onto poly-Lys-coated slides and fixed with cold methanol. Cells were stained using antibodies against MNK and secretory granule proteins DBM and CGA. The nucleus (arrow) and secretory granules (arrowhead) are indicated.

in Fig. 27, adrenal chromaffin (top panel) and PC12 cells (lower panel) stained with MNK antibody exhibited a punctate vesicular staining pattern throughout the cytosol (arrowheads), that was excluded from the nucleus (long arrow). The pattern of MNK staining in both cell types was similar to the staining observed for the secretory granule proteins DBM and CGA. Since it has been previously shown that the punctate staining in these cells correspond to secretory granules...
(Oyarce and Eipper, 2000) our findings suggest that MNK is localized to secretory granules in addition to TGN in adrenal chromaffin and PC12 cells.

Figure 28. Trafficking of MNK to the plasma membrane following stimulation of exocytosis in PC12 cells.

Figure 28. PC12 DBM cells were incubated in PBS containing 100 μM KCl to stimulate exocytosis of secretory granules for 5, 10, or 20 min at 37°C. Control cells (0 min) were incubated in PBS without KCl for 20 min. Following treatment, the cells were fixed, as described in Materials and Methods, and incubated with the MNK antibody. Punctate vesicular staining that resemble secretory granules are depicted with the arrowhead (SG), while the plasma membrane is illustrated by the short bold arrow (PM). The long arrow points to the nucleus of the cell.
To further confirm the localization of MNK to secretory granules in PC12 cells, we performed studies where exocytosis of secretory granules was stimulated in PC12 cells by depolarization. During exocytosis, secretory granules fuse with the plasma membrane exposing membranous proteins to the plasma membrane, while the soluble content is released into the extracellular space. For these studies we used KCl, a depolarizing agent, to stimulate exocytosis in PC12 cells and the presence of MNK was analyzed by immunofluorescence microscopy and surface biotinylation (Milgram et al., 1992; Oyarce and Eipper, 2000; Cavadas et al., 2002; Relman et al., 2006. Because it was determined that MNK behaves similarly in adrenal and PC12 cells, subsequent experiments were carried out in PC12 cells because they are easier to manipulate and maintain in culture. To visualize the trafficking of MNK from secretory granules to the plasma membrane, PC12 cells were stained with the MNK antibody following depolarization of the cells with KCl, for 5, 10, and 20 min (Fig. 28). In control cells incubated without KCl treatment (0 min), MNK was observed in punctate vesicular staining throughout the cytosol with the nucleus devoid of MNK staining, as previously shown (arrowheads, SG). Most importantly, no significant MNK staining was observed at the plasma membrane in control cells. However, there was an enhancement of MNK staining at the plasma membrane after depolarization of the PC12 cells with KCl (bold arrow, PM). It is important to point out that the punctate vesicular staining still observed throughout the cytosol after stimulation is due to MNK in secretory granules that have not been stimulated for secretion, as normally happens in endocrine cells.
Figure 29. Movement of DBM to the plasma membrane following exocytosis of PC12 cells

To ensure that KCl is, in fact, stimulating secretory granules exocytosis, the trafficking of DBM, a resident secretory granule protein also was analyzed (Fig. 29). Following KCl treatment for 10 min there was an increase of DBM immunostaining at the plasma membrane in PC12 cells. Therefore the movement of MNK and DBM to the plasma membrane following exocytosis indicate that MNK is found in secretory granules in endocrine PC12 cells. In order to further establish the localization of MNK to secretory granules, surface biotinylation and Western blot analysis were performed after stimulation of PC12
cells with KCl. PC12 cells were stimulated with 10 mM KCl for 5, 10, and 20 min and membrane proteins were biotinylated as described in Methods. Western blot analysis using the MNK antibody showed that a basal level of MNK biotinylation was observed in control cells indicating that some MNK is present at the plasma membrane at steady state (Fig. 30A). Following stimulation of regulated secretion with KCl for 5, 10, and 20 min, an increase in MNK biotinylation was observed compared to the control. This indicates an increase of MNK at the plasma membrane following fusion of secretory granules. It is also observed that after 10 min of stimulation with KCl, there is more of an enrichment of MNK at the plasma membrane in comparison to the other time points. This finding is consistent with the immunostaining of MNK following KCl treatment. This data could represent a saturation of secretory granule fusion at the plasma membrane as no further increase in MNK biotinylation was observed beyond 10 min with KCl stimulation.

Quantification of MNK biotinylation indicated a 2.4 +/- 0.06 fold (P < 0.0001) increase in MNK cell surface biotinylation after 5 min of stimulation, while a 3.8 +/- 0.2 fold increase (P < 0.0001) was observed after 20 min treatment (Fig. 30B). Interestingly, a maximum increase of 5.4 +/- 0.25 fold increase (P < 0.0001) in MNK biotinylation was reached after 10 min of stimulation (Fig. 30B). The increase in MNK biotinylation as well as MNK immunostaining indicate an increase in MNK trafficking to the plasma membrane following exocytosis of secretory granules containing MNK.
Figure 30. Re-localization of MNK following KCL stimulation of PC12DBM cells

A

Time (min): 0 5 10 20

B

Cell Surface MNK (Fold Increase) 0 2 4 6

Figure 30. A.) PC12DBM cells were stimulated with 10 mM KCl for 0, 5, 10, and 20 minutes and cell surface proteins were biotinylated as described in methods. Biotinylated proteins were fractionated by SDS-PAGE and Western blot analysis was done using the MNK antibody. B.) Quantification of biotinylated MNK following KCl stimulation. Histogram illustrates the relative fold increase compared to control of MNK at the cell surface following depolarization. The asterisk indicates statistically significant differences between cell surface MNK proteins from treated and control cells with P < 0.0001 (*) and are reproducible as similar results were obtained in four independent experiments.
**Trafficking of MNK in Response to Copper Levels in PC12 cells.** Previous studies in MNK transfected fibroblasts have shown that MNK is localized to the TGN in these cells and it redistributes to the plasma membrane when exposed to elevated copper levels for copper efflux. While, in decreased copper levels, MNK returns to the TGN (LaFontaine et al., 1988a; Camakaris et al., 1995; Camakaris et al., 1999; Pase et al., 2004). Although copper-dependent trafficking of MNK in fibroblasts has been well established, little is known about how MNK behaves in response to copper in endocrine cells that not only express endogenous MNK and contains copper requiring enzymes, but has a unique subcellular localization of MNK. Therefore, MNK trafficking in response to copper levels was analyzed in PC12 cells by differential sucrose density gradient fractionation, immunofluorescence microscopy, and cell surface biotinylation experiments.

In order to analyze MNK trafficking at low copper levels, PC12 cells were incubated with 50 μM BCS, a copper chelator, for 16 h and then subjected to subcellular fractionation. In the P2 sucrose density gradient, MNK showed a similar bimodal distribution between MNK in the control and BCS-treated cells (Fig. 31). However, it seems that following BCS treatment there is more MNK in fractions 8-11, which are enriched in the TGN marker, Syn6. Although there is a possibility that the increase in MNK in fractions 8-11 is due to an effect of BCS on the level of MNK protein PC12 cells, this is unlikely based on the fact that BCS decreased MNK levels in AtT-20 cells, another endocrine cell line (Dr. Tami Steveson; personal communication). Most importantly, the organelle markers DBM, CGA, p38, and Syn6 showed a distribution pattern in the presence of BCS
Figure 31. PC12 DBM cells were incubated with 50 μM BCS for 16 h and subjected to differential fractionation to obtain P2 pellet. The P2 pellet was resuspended in 0.32 M sucrose and further fractionated on a P2 sucrose density gradient containing BCS. Fractions taken from top to bottom of the gradient were fractionated on SDS-PAGE and the distribution of MNK and organelle markers DBM, CGA, p38, and Syn6 was analyzed by Western blot. The distribution of proteins were compared to that of control gradient.

that is similar to the one observed in the control P2 sucrose gradient (Fig. 25). These data indicate that the organelle markers did not undergo a change in subcellular distribution after BCS treatment. When the P3 gradient obtained from
Figure 32. Sucrose gradient fractionation of P3 pellet following BCS treatment

PC12 DBM cells were incubated with 50 μM BCS for 16 h and subjected to differential fractionation to obtain P3 pellet. The P3 pellet was resuspended in 0.32 M sucrose and further fractionated on a P3 sucrose density gradient containing BCS. Fractions taken from top to bottom of the gradient were fractionated on SDS-PAGE and the distribution of MNK and organelle markers DBM, CGA, p38, and Syn6 was analyzed by Western blot. The distribution of proteins were compared to that of control gradient.

PC12 cells incubated in BCS was compared to the control, significantly more MNK was observed in fractions 4-7 enriched primarily in the TGN marker Syn6 (Fig. 32). The fact that no re-distribution of the organelle markers DBM, CGA, p38, and Syn6 was observed indicates that the shift in the MNK distribution...
Figure 33. PC12 DBM cells were incubated with 200 μM CuCl₂ for 5 hours and the P2 pellet obtained by differential fractionation was resuspended in 0.32 M sucrose and further fractionated on a P2 sucrose density gradient containing CuCl₂. The P2 gradient contained layers of sucrose from 0.3 M to 1.6 M. Equal aliquots of the 14 fractions taken from top to bottom of the gradient were fractionated on SDS-PAGE and Western blot analysis were performed to determine the distribution of MNK and organelle markers using the MNK, DBM, CGA, p38, and Syn6 antibodies.

pattern was specific and only affected the MNK transporter, as expected. The results obtained following treatment of PC12 cells with BCS suggest a re-distribution of MNK to the TGN seen as a greater enrichment of MNK in fractions.
Figure 34. Sucrose gradient fractionation of P3 pellet following CuCl$_2$ treatment

![Sucrose Gradient Fractionation](image)

Figure 34. PC12 DBM cells were incubated with 200 μM CuCl$_2$ for 5 h and the P3 pellet obtained by differential fractionation was resuspended in 0.32 M sucrose and further fractionated on a P3 sucrose density gradient containing CuCl$_2$. The P3 gradient contained layers of sucrose from 0.3 M to 2.2 M. Equal aliquots of the 14 fractions taken from top to bottom of the gradient were fractionated on SDS-PAGE and Western blot analysis were performed to determine the distribution of MNK and organelle markers using the MNK, DBM, CGA, p38, and Syn6 antibodies.

containing Syn6. Interestingly, when PC12 cells were incubated with 200 μM CuCl$_2$ for 5 h to mimic copper overload, the P2 and P3 sucrose density gradient exhibited a re-distribution of MNK to lighter fractions (Fig. 33). This re-distribution was best seen in the P2 density gradient where a higher amount of
MNK was observed in fractions 8-11. It is important to point out that increasing levels of copper did not change the distribution of organelle markers in P2 and P3 sucrose density gradients as compared to control gradients (Fig. 25 and 26). Although the distribution of a plasma membrane marker was not analyzed in the P2 and P3 gradients, previous studies in fibroblasts have shown that MNK redistributes to the plasma membrane from the TGN with increasing copper levels. Because of the limitations that the sucrose gradients have to analyze MNK trafficking, further studies were developed using immunofluorescence microscopy and cell surface biotinylation.

**Analysis of copper-dependent trafficking of MNK by immunofluorescence microscopy.** Our data obtained by sucrose density gradient fractionation of PC12 cells treated with BCS and copper chloride showed that MNK undergoes a change in its distribution pattern. As a result of the treatment, however, it is not clear that this re-distribution is associated with a change in subcellular localization of MNK in response to changes in copper levels. Therefore, immunofluorescence microscopy was utilized in order to visualize MNK trafficking in response to changes in copper levels. Control PC12 cells incubated without BCS or copper chloride showed the typical MNK punctate vesicular staining pattern throughout the cytosol that resembles the staining of secretory granule markers DBM and CGA (Fig. 28) indicating the presence of MNK in secretory granules (Fig. 35; arrowhead, SG). However, in PC12 cells treated with BCS, MNK stained in the perinuclear region, a staining pattern distinctive of TGN. As
Figure 35. Re-localization of MNK in PC12 DBM cells following treatment with BCS or CuCl$_2$

Figure 35. PC12DBM cells treated with 50 μM BCS for 16h or 200 μM CuCl$_2$ for 5 h were fixed in cold methanol and immunostained using the MNK antibody. MNK staining in treated cells was compared to that of control PC12DBM cells. The secretory granules are identified with the arrowhead (SG) while the TGN is seen at the perinuclear region depicted by the short arrow (TGN). The plasma membrane is identified by the short bold arrow (PM). The long arrow points to the cell nucleus.

expected, vesicular staining throughout the cytosol was still seen after BCS treatment. Furthermore, PC12 cells treated with copper chloride showed MNK staining at the periphery of the cell that resembled the staining of plasma membrane markers (short bold arrow, PM) (LaFontaine and Mercer, 2007; Petris and Mercer, 1999). MNK staining was excluded from the nucleus of control and treated PC12 DBM cells. Our data demonstrate the trafficking and accumulation of MNK to the TGN at lower copper levels following BCS treatment, while an increase in copper levels relocalized MNK to the plasma membrane in PC12 DBM cells.
Analysis of copper-dependent trafficking of MNK by biotinylation. To further analyze the re-localization of MNK to the plasma membrane, PC12 cells were incubated in with increasing copper concentrations of 25, 50, 100, 200, and 400 μM for 5 hours and compared to control cells incubated in medium without copper. Following treatment, cell surface proteins were biotinylated and MNK was identified by Western blot analysis. As shown in Fig. 35A, a basal level of MNK biotinylation was observed in control cells, indicating the presence of MNK at the plasma membrane at steady state. However, an increase in MNK biotinylation at the cell surface was observed with increasing concentration of copper. This increase in MNK biotinylation in response to copper levels indicates an increase in trafficking of the MNK protein to the plasma membrane.

Quantification of the MNK cell surface biotinylation (Fig. 35B) showed an increase of 1.5 +/- 0.35 fold (P < 0.03) and 1.9 +/- 0.32 fold (P < 0.0005) in MNK biotinylation following incubation of PC12 DBM cells with 100 μM and 200 μM copper chloride, respectively. A maximum of 2.3 +/- 0.41 fold (P < 0.0001) increase in MNK at the plasma membrane was observed after treating the cells with 400 μM copper chloride for 5 h. Interestingly, no significant increase in MNK biotinylation was observed at copper concentrations lower than 100 μM copper chloride. No further increase was observed at 500 μM copper chloride indicating that MNK amount at the plasma membrane reached saturation when copper levels exceed 400 μM. It is important to point out that approximately 5% and 7% of the total MNK in the cell extract was biotinylated at 200 μM and 400 μM of
Figure 36. Re-localization of MNK to the plasma membrane in response to increasing copper levels

A

B

Figure 36. A.) PC12 DBM cells were incubated with increasing concentrations of CuCl$_2$ for 5 h. Control cells were incubated in medium without CuCl$_2$. Cells were biotinylated, extracted in TMT and biotinylated proteins were isolated using NeutrAvidin beads. Biotinylated proteins were fractionated by SDS-PAGE and MNK was detected by Western blot analysis. B.) Quantification of biotinylated MNK was determined using BioRad GS-670 Imaging Densitometer. Asterisks indicate statistically significant differences between MNK cell surface biotinylation from treated and control cells with $P < 0.03$ (*), $P < 0.0005$ (**), and $P < 0.0001$ (***) . Similar results were obtained with four independent experiments.

copper chloride, respectively. Thus, a small percentage of the total amount of MNK present in the cell responded to changes in copper levels. In order to
determine that the increase in MNK cell surface biotinylation was due to an increase in MNK trafficking to the plasma membrane rather than due to an increase in levels of MNK protein, the total amount of MNK protein was determined in cell extracts from biotinylated PC12 cells treated with 0, 50, and 400 μM copper chloride for 5 hours. As shown in Fig. 37, there is not significant change in the amount of MNK protein present in the treated samples compared

Figure 37. Western blot analysis of biotinylated and total MNK protein following CuCl₂ treatment of PC12DBM cells

<table>
<thead>
<tr>
<th>CuCl₂ μM:</th>
<th>0</th>
<th>50</th>
<th>400</th>
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<tbody>
<tr>
<td>Biotinylated MNK</td>
<td><img src="image1" alt="Biotinylated MNK" /></td>
<td><img src="image2" alt="Biotinylated MNK" /></td>
<td><img src="image3" alt="Biotinylated MNK" /></td>
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<tr>
<td>Total MNK protein</td>
<td><img src="image4" alt="Total MNK protein" /></td>
<td><img src="image5" alt="Total MNK protein" /></td>
<td><img src="image6" alt="Total MNK protein" /></td>
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Figure 37. In the top panel, PC12DBM cells were treated with either 50 μM or 400 μM CuCl₂ for 5 h and followed by biotinylation of cell surface proteins. Cells were extracted in TMT and an equal amount of protein for each treatment condition was incubated with NeutrAvidin beads. Biotinylated proteins were fractionated by SDS-PAGE and MNK analyzed by Western blot. Cell extract from untreated and biotinylated PC12 DBM cells is used as control. In the bottom panel, equal amount of total protein from PC12 DBM cells treated with 50 μM or 400 μM CuCl₂ for 5 h was fractionated by SDS-PAGE and Western blot analysis was performed using the MNK antibody. Untreated PC12 DBM cell extract was used as a control.
to the control that can account for the 2.3-fold increase in MNK biotinylation. Therefore, these data indicate that the increase in MNK biotinylation was due to an increase in MNK trafficking to the plasma membrane in PC12 cells in response to increasing copper levels.

Because we have already demonstrated that MNK is localized to two distinct compartments, the secretory granules and TGN in PC12 cells, it was important to determine that trafficking of MNK to the plasma membrane in response to copper levels occurred from the TGN and not from secretory granules. For these studies, PC12 cells were biotinylated following incubation with 200 μM of copper chloride for 5 hours and Western blot analysis was performed using antibodies against MNK and the secretory granule protein, DBM. As expected, the DBM antibody did not detect any biotinylated DBM in response to increasing copper levels (Fig. 38). The lack of DBM biotinylation indicates that the trafficking of protein from secretory granules is not influenced by copper levels. Therefore, MNK trafficking in response to copper levels originates from the TGN as previously shown for fibroblasts (Camakaris et al., 1995; Camakaris et al., 1998a; Camakaris et al., 1999).
Figure 38. Cell surface biotinylation of MNK and DBM in PC12 DBM cells treated with CuCl₂

![Image of Western blot](image)

Figure 38. PC12DBM cells were incubated with medium containing 200 μM CuCl₂ for 5 h followed by cell surface biotinylation. Cell extract was fractionated by SDS-PAGE and biotinylated MNK (top panel) and DBM (bottom panel) were analyzed by Western blot.

To analyze the rate at which MNK moves from the TGN to the plasma membrane, PC12 cells treated with 200 μM copper chloride for 0, 5, 15, 30, 60, 120, 180, 240, 300 min, followed by cell surface biotinylation of proteins. Western blot analysis of MNK showed an increase in MNK biotinylation at the plasma membrane with increasing time to copper exposure (Fig. 39A). The quantification of the time course of MNK biotinylation (Fig. 39B) indicated that a significant 1.4 +/- 0.29 fold (P < 0.002) increase in MNK biotinylation occurred after 1 hour of treatment. Interestingly, a lag time of 3 hours was observed
Figure 39. Time-dependent re-localization of MNK in response to increasing copper levels in PC12 cells

A

Time (min): 0 5 15 30 45 60 120 180 240 300

MNK

B

Cell Surface MNK (Fold Increase) vs. Time (min)

Figure 39. A.) PC12 DBM cells were incubated with 200 μM of CuCl₂ for 0, 5, 15, 30, 45, 60, 120, 180, 240, and 300 min. Control cells were incubated in medium without CuCl₂. Cells were biotinylated, extracted in TMT and biotinylated proteins were isolated using NeutrAvidin beads. Biotinylated proteins were fractionated by SDS-PAGE and MNK was detected by Western blot analysis. B.) Quantification of biotinylated MNK was determined using BioRad GS-670 Imaging Densitometer. Asterisks indicate statistically significant differences between MNK biotinylation from treated and control cells incubated at various time points with P < 0.002 (*) and P < 0.0001 (**). Similar results were obtained with four independent experiments.
before reaching a 2.0 +/- 0.08 fold (P < 0.0001) increase in MNK biotinylation compared to the control. This maximum increase in MNK biotinylation is consistent with data obtained in the dose response experiments (Fig. 31). Overall, our data indicate that MNK trafficking to the plasma membrane in response to increasing copper levels occurs within 1-3 h of copper exposure.
CHAPTER V: DISCUSSION (Part 1)

Neuroendocrine cells are set apart from other cells in the fact that they have two different secretory pathways; the constitutive pathway and the regulated secretory pathway (Sossin et al., 1990). Many questions exist as to what the mechanism is regarding a protein’s destination to either pathway. It has been hypothesized that the sorting of constitutive and regulated proteins is a dynamic process that requires the presence of a sorting signal on proteins destined for secretory granules (Miller and Moore, 1990).

DBM is unique amongst the catecholamine biosynthetic enzymes in its subcellular localization because it is the only enzyme in this biosynthetic pathway located in the secretory granule lumen of adrenal chromaffin cells and adrenergic neurons (Stewart and Klinman, 1988; Oyarce and Eipper, 2000). There have been few studies done in order to try and elucidate the structure and/or function of the various domains of DBM. For instance, it has been shown that when the DBM signal sequence, found at the amino terminus of DBM, was appended to the soluble PHM to form the DBM signal PHMs chimera, the PHM protein changed its localization from soluble to membrane bound (Oyarce and Eipper, 2000). This data suggests that the DBM signal sequence contains structural information necessary for membrane anchoring. Because the DBM domains containing routing information for sorting DBM to secretory granules have not been elucidated, we were interested in analyzing the structural and/or sorting information present in the carboxy-terminus of DBM. For these studies, we
appended the carboxy-terminus of DBM to the PHM domain of PAM and stably expressed this chimera in AtT-20 cells. PHMs/DBMCT was analyzed by immunofluorescence microscopy, metabolic labeling, subcellular fractionation, and Western blotting.

The expression of PHMs/DBMCT in AtT-20 cells showed two molecular species consisting of a 51 kDa and 48 kDa forms (Fig. 8) and both of these forms are larger in size compared to the 38 kDa PHM protein. The size difference between PHMs/DBMCT and PHM indicates the presence of the DBMCT attached to the PHMs protein. Interestingly, the subcellular distribution of PHMs/DBMCT differed from that of PHMs alone as subcellular fractionation experiments demonstrated that the PHMs/DBMCT exists in soluble and membrane fractions, while PHM was mostly found in the soluble fraction with very little present in the membrane fraction (Fig. 9). It is quite clear that with the attachment of the DBMCT to PHMs there was an increase in PHMs membrane association. To investigate this observation further, membranes were treated with sodium carbonate, a technique used to dissociate peripheral proteins (Oyarce et al., 2001) in order to analyze the membrane association of PHMs/DBMCT (Fig. 10). However, after the carbonate wash treatment, which because of its high pH causes non-covalently bound proteins to become dissociated from the plasma membrane, the PHMs/DBMCT was still found in the particulate fraction. While very little PHMs was found in the membrane fraction initially, it was entirely removed from the membrane following the high pH carbonate wash. This observation is consistent with previous studies that
showed PHM association with secretory granule membranes in anterior pituitary (Oyarce and Eipper, 1995). These data suggest that when DBMCT is attached to PHMs, a significant amount of the chimera is membrane associated in secretory granules.

Although previous studies have indicated that DBM is a type II integral membrane protein (Oyarce et al., 2001) due to anchoring properties it its signal sequence, it is not known whether this domain is responsible for membrane attachment in the full length DBM protein. Furthermore, previous studies aimed at elucidating the mechanism by which the full length DBM anchors to the secretory granule membranes have excluded glycosyl phosphatidylinositol tail (Stewart and Klinman, 1988) or noncovalent association with phosphatidylserine (Bon et al., 1991) as possible mechanisms. Another interpretation of our data could be that, just like the DBM signal sequence, the DBMCT domain could also have anchoring properties for membrane association. This would not be the first time that a secretory granule protein, present in both soluble and membrane-associated forms, uses its COOH-terminal region as a membrane anchor. For example, the protein Carboxypeptidase E (CPE), an enzyme that is involved in the biosynthesis of many peptide hormones and neurotransmitters that also exists in soluble and membrane bound forms (Fricker and Snyder, 1982) secretory granules in neuroendocrine cells. Studies using synthetic peptides that correspond to the COOH-terminal region of CPE as well as fusion proteins containing 51 amino acids of the COOH-terminal appended to albumin have demonstrated that its COOH-terminus is involved in anchoring CPE to granule
membranes (Fricker and Angeletti, 1990; Mitra et al., 1994). Furthermore, studies using CPE mutants with COOH-terminal deletions showed that while the COOH-terminal is involved in membrane binding, it also has a distinct region involved in the intracellular trafficking of CPE to secretory granules in AtT-20 cells (Varlamov, 1995). Thus, it is quite possible that the DBMCT may very well play a role in the ability of DBM to anchor to secretory granule membranes. Until additional studies are developed to elucidate whether the DBMCT acts as a membrane anchor, this possibility cannot be excluded.

Because PHMs/DBMCT exists as two molecular species in AtT-20 cells, metabolic labeling experiments were performed in order to investigate the synthesis and processing of the chimera. As shown in Fig. 11 it is apparent that the PHMs/DBMCT protein was initially synthesized in the 51 kDa form. However, after a 4 h chase period, the 48 kDa form was observed in the cells. The fact that the smaller second form appears 4 h after the protein was initially synthesized suggests that PHMs/DBMCT undergoes an endoproteolytic processing step in AtT20 cells. We can hypothesize that the site of cleavage is found in the COOH-terminal of DBM since PHMs is synthesized and secreted as one molecular species (Oyarce et al., 2001).

In order to further explore whether PHMs/DBMCT undergoes proteolytic processing in the DBMCT domain, Western blot analysis was performed using both, the PHMs and the rhodopsin antisera. Our data showed that the PHMs antibody was able to detect both forms of the chimeric protein, while the rhodopsin antibody could only detect the 51 kDa form (Fig 12A). The fact that
the 48 kDa form was not detected with the rhodopsin antibody indicates that after the proteolytic processing step takes place, a fragment of the DBMCT containing the rhodopsin tag was lost. Because the size difference between the two molecular species is 3 kDa, we estimate that this difference corresponds to approximately 30 amino acids. Interestingly, analysis of the PHMs/DBMCT amino acid sequence revealed the presence of a putative cleavage site in the DBMCT (Fig. 12B). This potential site contains an arginine as indicated by the sequence RXXR, where “R” is an arginine residue that is separated by two other amino acids.

Studies have shown that the RXXR sequence is the site of cleavage by various cellular proteases, including Furin and the prohormone convertases PC1/3, PC2, and PC4 that are mammalian homologues to the yeast endoprotease, Kex2 (Fuller et al., 1989; Hakes et al., 1991; Nakayama et al., 1992). Northern blot and in situ hybridization experiments have shown that the PC2 and PC1/3 proteases are only found in neuroendocrine tissues and cell lines such as AtT-20 cells (Hakes et al., 1991), while Furin is found in many tissues, including the neuroendocrine system, liver, gut and brain (Fuller et al., 1989; Hakes et al., 1991). PC4 convertase is only found in the testis (Nakayama, 1992). Although many cellular processes including intracellular protein trafficking as well as endocrine and neural functions are regulated by the proteolysis of proteins (Trifaro, 1977; Nakayama et al., 1992; Sudhof, 1995), it is not known whether or not any one of these proteases are responsible for the endoproteolytic cleavage that occurs in the DBMCT. It is important to point out
that these proteases function within the secretory pathway (Eipper et al., 1993; Zhou and Bloomquist, 1993) where Furin is localized in the TGN and small secretory vesicles while the convertases PC2 and PC1/3 are found within dense core secretory vesicles where they process on prohormone and neuropeptide precursors (Bloomquist et al., 1991; Seidah and Chretien, 1992). This proteolytic step in the secretory pathway is essential for the bioactive function of hormones and peptides.

Since the role of proteolytic processing in DBMCT is unclear, it was important to determine whether or not this cleavage step takes place in the full length DBM protein. For these studies, we appended a myc tag to the COOH-terminus of the full length DBM protein and transfected it into PC12 cells. The PC12 cell line was chosen for expression of the DBM-myc because previous studies have shown that despite the fact that AtT-20 cells have regulated granules, exogenous DBM was not stored in these organelles. Thus, DBM undergoes a cell-type specific storing and trafficking to secretory granules (Oyarce and Eipper, 2000). The fact that the DBM antibody detected the DBM-myc protein, while the myc antibody was not able to detect the DBM-myc protein suggests that in PC12 cells, the full length DBM protein undergoes an endoproteolytic processing in its COOH-terminus. Thus, the cleavage site present in the DBMCT is not masked as the full length DBM is folded and assembled and is recognized by a protease in the secretory pathway. Most importantly, the fact that this cleavage took place in PC12 as well as AtT-20 cells suggests that the proteolytic cleavage of DBMCT is not cell type specific.
Although the significance of the DBMCT processing is unknown, it is possible that this cleavage step is necessary for routing of DBM to secretory granules. On the other hand, it is possible that this cleavage is important for the overall DBM structure and folding configuration which are important for the maturation of DBM as it proceeds through the secretory pathway. It would be interesting to determine whether or not site-directed mutagenesis of this cleavage sequence would alter DBM localization to secretory granules or would change the ability of DBMCT to have partial membrane association.

Since the proteolytic processing of DBMCT most likely takes place en route through the secretory pathway, we wanted to determine the subcellular localization of the chimeric protein. Thus, PHMs/DBMCT subcellular localization in AtT-20 cells was investigated using immunofluorescence microscopy and stimulation of exocytosis. Immunostaining of PHMs/DBMCT cells showed staining in the tips of cellular processes where secretory granules are located and also a diffuse staining that resembles the ER. Moreover, double immunostaining showed PHMs/DBMCT co-localized with ACTH in secretory granules and the Bip protein in the ER (Fig. 15). The ER localization differs from the secretory granule and TGN staining seen in AtT-20 PHMs (Fig. 13). PHM localization is in agreement with previous results in AtT-20 cells (Milgram et al., 1992).

To further confirm the presence of PHMs/DBMCT in secretory granules, exocytosis of secretory granules was stimulated in AtT-20 cells. The ability to stimulate cell secretion with a secretagogue can validate the chimera’s
localization because secretory granules when stimulated fuse with the cell membrane and release its contents. The fact that there is a 2.3-fold (149 +/- 5.9 ARB, P< 0.0001) increase in PHMs/DBMCT chimera secreted into the media followed by stimulation of cells with BaCl₂ is an indication of the presence of PHMs/DBMCT in secretory granules in AtT-20 cells (Fig. 16). Taken together our data indicate that the PHMs/DBMCT chimera is localized to secretory granules and ER. Interestingly, stimulation of PHMs/DBMCT cells generated a 1.6-fold (88 +/- 16.9 ARB, P < 0.0004) increase in the 51 kDa form, while the secretion of the 48 kDa form had a 2.3-fold (149 +/- 5.9 ARB, P< 0.0001) increase. The difference in the amount of secretion between both forms could be explained by the fact that the endoproteolytic cleavage in the DBMCT that originates the 48 kDa form confers a more soluble characteristic to the 48 kDa form when compared to the 51 kDa form.

Prior studies have shown that PHMs when expressed by itself in AtT-20 cells localizes to secretory granules (Milgram et al., 1992). Therefore, although PHMs/DBMCT is found in secretory granules, it is difficult to evaluate the sorting and trafficking information in DBMCT when it is attached to the PHMs protein. Thus, it was important to isolate the DBMCT in order to answer the question of whether it contains trafficking information. To find out whether DBMCT has trafficking information to granules, we attached this domain to the reporter molecule, EGFP, to generate the EGFP/DBMCT chimera which was expressed in PC12 cells. EGFP was chosen as a reporter molecule because it’s fluorescent properties and the fact that it is not known to have any intrinsic trafficking and
sorting properties (Davenport and Nicol, 1955). In PC12 cells, the EGFP/DBMCT chimera showed a non-specific and diffuse staining pattern throughout the cytoplasm and did not localize to secretory granules (Fig. 18). It is possible that one of the reasons as to why the chimera is not localized to secretory granules is because the chimera was not folded properly and it remained in the ER. However, another explanation as to why the EGFP/DBMCT chimera does not route to granules could be because alone, it is not sufficient for sorting EGFP to secretory granules suggesting that other domains in DBM, along with the DBMCT domain, could be necessary for trafficking of DBM to secretory granules. Since DBM contains several cysteine residues that form interchain and intrachain disulfide bonds, it is possible that the formation of disulfide bonds between DBMCT and the putative catalytic core and/or the pro-region is important in order to sort DBM to its place of residence in secretory granules in neuroendocrine cells.
Copper is an essential element that is required for all organisms to survive (Harris, 2003). Copper has the capability to take on distinct redox states where it can be oxidized as Cu$^{2+}$ or reduced to the Cu$^{1+}$ form in order to participate in chemical reactions by serving as a cofactor for many cuproenzymes (Hartman and Evenson, 1992). Clearly, a deficiency in copper has grave consequences as the activity of many essential enzymes are altered. For example, ATP shortage, myopathy, ataxia, and seizures can be a result of a copper deficiency in Cytochrome c oxidase (Sparaco et al., 1993), while copper deficiency in lysyl oxidase can cause connective tissue disorders and vascular rupture (Kodama et al., 1999). Whereas copper is essential for the function of cuproenzymes, excess copper is highly toxic and its homeostasis must be tightly regulated by chaperones and copper transporters such as MNK, a P-type ATPase that plays a role in copper efflux. Although MNK localization have been examined in fibroblasts expressing exogenous MNK, little is known about the subcellular localization and trafficking of MNK in endocrine tissues that endogenously express MNK and cuproenzymes. Therefore, adrenal chromaffin cells and its PC12 tumor cell line were used to evaluate the role of MNK in an endocrine cell system that contain the cuproenzyme DBM, synthesize catecholamines, and endogenously express MNK. Hence, we attempt to characterize the endogenous MNK protein in adrenal chromaffin and PC12 cells by investigating its subcellular localization and trafficking. For these studies, subcellular localization of MNK
was analyzed by differential fractionation and sucrose density gradient fractionation coupled to Western blot analysis and immunofluorescence microscopy. Furthermore, MNK subcellular localization in these neuroendocrine cells was confirmed with stimulation with a secretagogue. The trafficking of MNK in response to copper levels was also analyzed by incubating PC12 cells with copper or a copper chelator to stimulate a copper-dependent trafficking to the plasma membrane and/or TGN. The localization of MNK was determined by cell surface biotinylation, immunofluorescence microscopy, and Western blot analysis. The experiments done here have shown that in adrenal chromaffin and PC12 neuroendocrine cells, the subcellular localization of MNK is TGN and secretory granules. Our results also indicate that MNK undergoes a copper-dependent trafficking where it re-distributes to the plasma membrane with increasing copper and moves back to the TGN under low copper conditions.

While immunostaining of MNK in chromaffin and PC12 cells showed primarily a punctate vesicular pattern indicative of secretory granules with no significant staining in the TGN (Fig. 19), the pellet fractions obtained from differential centrifugation, which separates organelles on the basis of size, demonstrated the co-distribution of MNK and the TGN marker Syn 6 in the P2 and P3 pellets derived from both cell lines (Fig. 21 and 22). Furthermore, when the P2 pellet derived from adrenal chromaffin cells was further fractionated on a sucrose density gradient, which separates organelles on the basis of their density, MNK exhibits a bimodal distribution in lighter fractions 6-9 and heavier fractions 12-14 (Fig. 23). Interestingly, in the lighter fractions, MNK co-distributes
with fractions enriched in Syn 6. A similar distribution of MNK and Syn 6 was observed in the lighter fractions 4-8 in the P3 gradient (Fig. 24). Consistent with these findings in adrenal chromaffin cells was the data obtained from sucrose density gradients derived from PC12 cells. In the P2 gradient, MNK also had a bimodal distribution to lighter fractions 9-11 and heavier fractions 12-14 (Fig. 25). It is in the lighter fractions 8-11 that MNK co-distributes with the TGN marker, Syn 6, while in the P3 gradient MNK and Syn 6 co-distributed in the lighter fractions 4-8 (Fig. 25 and 26). The distribution of MNK to fractions that were enriched in the Syn 6 TGN resident protein suggests that MNK is localized to this organelle. Prior studies using non-endocrine cells have localized MNK to the TGN (Yamaguchi et al., 1996; Ackland et al., 1997; Dierick et al., 1997; LaFontaine et al., 1998b; Ackland et al., 1999). For example, sucrose density gradient fractionation of HeLa cells (immortal cervical cancer cells) had a distribution of MNK in similar fractions as those containing γ-adaptin and mannose-6-phosphate receptor, which are resident proteins of the TGN. Furthermore, confocal immunofluorescence studies confirmed the localization of MNK to the TGN in HeLa cells (Yamaguchi et al., 1996). The localization of MNK in HeLa cells is consistent with the intracellular distribution of MNK in CHO cells (LaFontaine et al., 1998b) as well as in fibroblasts from MNK patients (LaFontaine et al., 1998a).

It is important to point out that in addition to the co-distribution of MNK with a TGN marker, our sucrose gradient data also depicted MNK enrichment in fractions containing secretory granule proteins DBM, CGA, and p65, consistent with the immunostaining data (Fig. 19). These granule markers were used
because DBM is a resident copper-requiring enzyme of secretory granules (Oyarce and Eipper, 2000), while CGA is a major component of secretory granules in endocrine cells (Blaschko et al., 1967) and p65 is a resident granule protein thought to be involved in exocytosis (Tucker and Chapman, 2002). The bimodal distribution of MNK in the adrenal P2 sucrose gradient showed an enrichment of MNK in the lighter fractions 6-9 and heavier fractions 12-14, and it is in these heavier fractions that MNK co-distributes with the secretory granule proteins DBM, CGA, and p65 (Fig. 23). In the adrenal P3 gradient, MNK in the heavier fractions 8-11 co-distributed with DBM, CGA, and p65. Despite the fact that the P3 gradient was overloaded, as seen by the pelleting of secretory granules, it is clear that the secretory granule markers coincide with MNK distribution in fractions 8-11 (Fig. 24). The P2 gradient derived from PC12 cells similarly depicted MNK enrichment in the heavier fractions 12-14 co-distributing with DBM and CGA (Fig. 25). The P3 gradient also showed secretory granule markers co-distributing with MNK in the heavier fractions 9-11 (Fig. 26).

Interestingly, while the TGN marker Syn 6 co-distributes with MNK in the lighter fractions in all these gradients, this protein was found in heavier fractions since it has been shown to also reside in immature secretory granules (Bock et al., Wendler et al., 2001). The synaptic-like microvesicle marker p38 did not show significant overlap with MNK in the adrenal P2 gradient; however there is overlap between MNK and p38 in the heavier fraction 14 of the P2 gradient from PC12 cells and in the lighter fractions 5-7 of both the adrenal and PC12 cells P3 gradients (Fig. 25). It is not known whether MNK exists in synaptic-like
microvesicles in endocrine cells. Overall, our data obtained from immunostaining and subcellular fractionation studies suggests that MNK is localized to the TGN and secretory granules, a novel finding in endocrine cells. While it is known that MNK is localized to the TGN in transfected fibroblasts, recent studies have also shown MNK localization to secretory granules in acinar cells of the parotid gland, an exocrine cell system (D’Amico et al., 2005). In these studies, although MNK was found primarily in the TGN, it was suggested that MNK found in secretory granules could be functioning to secrete copper into saliva. The presence of MNK in secretory granules gives rise to the question of whether MNK is localized to these organelles because they are present in endocrine cells, and exocrine, or could it be due to the presence of copper-requiring enzymes such as DBM, in the secretory granules of adrenal and PC12 neuroendocrine cells. However when subcellular fractionation studies were performed in Wild-type PC12 cells containing undetectable amount of DBM, a similar distribution pattern was observed in the P2 and P3 gradient (data not shown).

Unique to these neuroendocrine cells is the fact that they contain regulated secretory granules that store catecholamines and peptides and they can be stimulated to fuse with the plasma membrane to release their contents. Thus, in order to confirm the presence of MNK in secretory granules, PC12 cells were depolarized by incubation with KCl and the presence of MNK at the plasma membrane was analyzed by immunostaining and cell surface biotinylation. In control cells incubated without KCl, MNK was observed in punctate vesicular staining throughout the cytosol and no significant MNK was observed at the
plasma membrane (Fig. 28). However, following KCl stimulation there was an enhancement of MNK at the plasma membrane (Fig. 28). Furthermore, quantitation of biotinylated MNK showed a significant increase of 5.4 +/- 0.25 fold (P < 0.0001) of MNK at the cell surface following KCl stimulation (Fig. 30). This data is consistent with an increase in immunostaining of MNK and membrane-bound DBM at the plasma membrane after stimulation with KCl (Fig. 29). Most importantly, the trafficking of membranous secretory proteins such as DBM to the plasma membrane in response to stimulation with secretagogues is evidence of the fusion of secretory granules with the membrane during exocytosis in neuroendocrine cells (Oyarce and Eipper, 1992; Milgram et al., 1992; Cavadas et al., 2002; Relman et al., 2006). Because the appearance of DBM at the plasma membrane indicates that exocytosis occurred in PC12 cells after depolarization with KCl, we can conclude that MNK appearance at the cell surface is a result of the fusion of secretory granules with the plasma membrane. Thus, our data confirms the localization of MNK to the secretory granules in PC12 cells.

Evidence exists that indicate that MNK is necessary to deliver copper to various cuproenzymes for function as well as to maintain copper homeostasis by participating in copper efflux when cells are in a state of copper overload. It is well known that in non-endocrine cells, MNK undergoes a copper-dependent trafficking from the TGN to the plasma membrane (LaFontaine et al., 1998; Camakaris et al., 1995; Camakaris et al., 1999; Pase et al., 2004). However, little is known about the behavior of MNK in neuroendocrine cells that contain endogenous MNK localized to secretory granules and TGN and have copper
requiring enzymes. Our immunostaining data indicate that MNK undergoes a re-
distribution in subcellular localization in response to copper levels (Fig. 35) where
PC12 cells exposed to high copper showed a significant increase in MNK
staining at the plasma membrane. However, when cells were incubated with a
copper chelator, MNK accumulated in the TGN. Additionally, cell surface
biotinylation of MNK following CuCl₂ treatment demonstrated a significant
increase of 1.9 +/- 0.32 fold (P < 0.0005) and 2.3 +/- 0.41 fold (P < 0.0001) of
MNK biotinylation at the plasma membrane following incubation of PC12 cells
with 200 μM and 500 μM CuCl₂, respectively (Fig. 36). Our data is consistent
with the trafficking of MNK in response to copper levels in non-endocrine cells
(Fig. 35).

Cell surface biotinylation was also used in order to investigate the rate at
which MNK re-distribution to the plasma membrane following exposure of PC12
cells to increasing copper levels (Fig. 39). It is apparent that there was a lag time
of 1 h before significant increase of 1.4 +/- 0.29 fold (P< 0.002) in MNK
biotinylation was observed at the plasma membrane after CuCl₂ treatment.
Moreover, it 3 h in order to reach the 2.0 +/- 0.08 fold (P< 0.0001) maximum
increase in MNK biotinylation following incubating PC12 cells with 200 μM CuCl₂.
Since there is no evidence that the trafficking of secretory granule resident
proteins respond to copper levels, we conclude that MNK trafficks from the TGN
to the plasma membrane and not from secretory granules in response to copper
levels. Most importantly, the lack of membrane-bound DBM biotinylation
following copper treatment (Fig. 38) confirmed that MNK trafficking in response to
copper levels originates from the TGN in neuroendocrine PC12 cells as previously shown in fibroblasts (Camakaris et al., 1995; LaFontaine et al., 1998; Camakaris et al., 1999; LaFontaine and Mercer, 2007). Although previous studies in MNK-transfected CHO cells have shown that with elevations in copper levels, there was an increase in MNK cell surface biotinylation (Pase et al., 2004), difference in the amount of MNK that re-distributed to the plasma membrane was observed in PC12 cells. For example, Pase et al. (2004) observed that after 100 μM and 500 μM CuCl2 treatment of CHO cells for 3 h, there is a 3.5-fold and 4-5-fold increase in MNK biotinylation, respectively while smaller increases of 1.5 +/- 0.35 (P< 0.03) fold and 2.3 +/- 0.41 (P<0.0001) fold were observed in PC12 cells. Interestingly, difference in the rate of MNK distribution between CHO and PC12 cells were observed. Pase et al. also observed a 2-fold and 3-fold increase in MNK biotinylation following 20 min incubation with 100 μM and 500 μM CuCl2, while a lag of 1.5 and 2 h was observed for the maximum 4-5 fold increase in biotinylation. In contrast, it took 3 h to observe a maximum 2.0 +/- 0.08 fold (P< 0.0001) increase in MNK biotinylation following treatment of PC12 cells with 200 μM CuCl2. It could be argued that the higher amount and rate at which MNK re-distributed to the plasma membrane in CHO cells is because MNK exogenously expressed in these cells is found in a higher quantity compared to the amount of endogenous MNK expressed in PC12 cells. Furthermore, it is important to point out that the differential subcellular distribution of MNK to secretory granules and TGN in PC12 cells could account for less MNK available to respond to increasing copper
levels. Studies have identified that after copper stimulation of MNK trafficking from the TGN, MNK diverts back to a rapid recycling pool, identified just beneath the plasma membrane, in fibroblasts, which participates in copper efflux (Pase et al., 2004). Thus, the presence of this rapid recycling pool may be responsible for the faster MNK re-distribution to the plasma membrane in fibroblasts. Since sorting and trafficking of MNK in endocrine cells are not well determined, additional studies will be needed to analyze MNK internalization in order to determine if such a system exists in PC12 cells. Lastly, another possible reason for such differences between PC12 cells and non-endocrine cells may lie in the cell type specificity of MNK behavior and kinetics of copper uptake, accumulation, and distribution.

Overall, our studies demonstrate that MNK is localized to the TGN as well as secretory granules in adrenal and PC12 neuroendocrine cells. The data also indicate that MNK re-distributes to the plasma membrane with increasing copper levels, and trafficks back to the TGN with low copper levels. It was observed that while MNK is found in TGN and secretory granules, it is the population of MNK in the TGN and not secretory granules that undergoes copper-dependent trafficking. Thus, the unique MNK localization to these organelles in endocrine cells suggests that there are distinctive roles for MNK in these compartments. It is reasonable to suspect that the population of MNK in the secretory granules functions to provide copper to the DBM cuproenzyme within the secretory granule. Recently, MNK was proven to be essential for the activity of tyrosinase, a cuproenzyme in the secretory pathway involved in melanogenesis (Petris et al.,
2000) and there is evidence for MNK to be important in the amidation of various peptides by the PAM protein (Steveson et al., 2003). Many of the previous studies pertaining to MNK's role in copper delivery may be attributed to its close proximity to cuproenzymes moving along the secretory pathway. However, the fact that MNK is found in secretory granules gives rise to the inquiry of how it may play a role in DBM function, a resident of these granules. Although it appears as though MNK in the TGN functions to regulate copper homeostasis, based on its role in copper efflux, that is not to say that the population of MNK in the TGN may also serve to supply copper to other copper-dependent enzymes in the secretory pathway. Furthermore, because the MNK protein is essential for neurodevelopment, MNK could also be involved in other possible functions promoting growth and maturity of neuroendocrine cell structure and function. Recent studies have implicated MNK in synaptogenesis and axonal outgrowth of olfactory sensory neurons and it was hypothesized that aberrant MNK protein contributes to neurodegeneration via the inability to promote axonal extension (El Meskini et al., 2007). In conclusion, neuroendocrine cells provide a unique and exceptional opportunity to expand our knowledge and better understand MNK function. Further studies are needed to assess the role of MNK in the synthesis and function of catecholamines in patients with Menkes disease.
CHAPTER VI: CONCLUSIONS (Part 1)

1.) PHMs/DBMCT is expressed in soluble and membrane-associated forms. A secreted form is also observed.

2.) Significant amount of PHMs changes its subcellular distribution from soluble to particulate when DBMCT is appended to PHMs to form the PHMs/DBMCT chimera.

3.) Endoproteolytic processing of DBMCT originates the smaller 48 kDa PHMs/DBMCT form in AtT-20 cells.

4.) Full length DBM undergoes endoproteolytic processing it is COOH-terminus in PC12 cells as shown by the inability to detect myc tag in PC12 DBM-myc.

5.) In AtT-20 cells, the PHMs/DBMCT chimera is localized to secretory granules and ER.

6.) The subcellular localization of PHMs/DBMCT to secretory granules was confirmed by stimulating exocytosis of AtT-20 cells with BaCl₂.

7.) EGFP/DBMCT expressed in AtT-20 cells shows diffuse ER staining with no staining in secretory granules. This indicates that DBMCT does not contain sufficient sorting and trafficking information to route DBM to secretory granules.
CHAPTER VI: CONCLUSIONS (Part 2)

1.) MNK is localized to TGN as well as secretory granules in adrenal chromaffin and PC12 cells indicating its unique localization in neuroendocrine cells.

2.) Following KCl stimulation of exocytosis in PC12 cells, an increase in the cell surface biotinylation of MNK confirms that MNK is localized to secretory granules.

3.) Trafficking of MNK in response to KCl stimulation is a novel finding. Our studies show for the first time that MNK trafficking can occur independent of copper.

4.) MNK re-distributes to the plasma membrane from the TGN with increasing copper levels.

5.) MNK trafficks from the plasma membrane back to the TGN with decreasing copper levels.

6.) The localization of MNK in neuroendocrine cells suggesting that MNK has sorting information for its routing to secretory granules.

7.) The localization of MNK in secretory granules and the TGN suggest different functions of MNK in these subcellular compartments.


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ABSTRACT

Dopamine β-monooxygenase (DBM) requires ascorbate, copper, and molecular oxygen to synthesize norepinephrine. Because little is known about the mechanism by which DBM is routed to catecholamine-containing secretory vesicles, we investigated the role of the DBM carboxy-terminus (DBMCT) by attaching it to the hydroxylating (PHM) domain of peptidyl-glycine α-amidating monooxygenase. The active PHMs/DBMCT chimera expressed in corticotrope AtT-20 cells was analyzed by Western blot, metabolic labeling, and immunostaining. The PHMs/DBMCT chimera contained a 48 and 51 kDa species, with the 48 kDa formed by proteolytic processing of DBMCT. Immunostaining showed that although PHMs/DBMCT is mainly localized to secretory vesicles in AtT-20 cells, it is also present in the ER. An EGFP/DBMCT chimera transiently transfected into pheochromocytoma PC12 cells showed ER staining, indicating that DBMCT does not contain sorting information for routing to secretory granules but rather it has a structural role. Although copper is essential for DBM function, excess copper is highly toxic and its homeostasis is tightly regulated by transporters and chaperones. Menkes copper transporter (MNK) is a P-type ATPase essential for an organism’s survival due to its role in regulating copper efflux. Mutations in MNK affect copper utilization by cuproenzymes involved in catecholamine and peptide biosynthesis, development and growth, thus causing Menkes syndrome that is characterized by mental retardation, neurodegeneration, connective tissue disorders and early childhood
death. Since little is known about MNK in neuroendocrine cells containing sequestered cuproenzymes, we investigated the localization and trafficking of endogenous MNK in chromaffin and PC12 cells where catecholamines are synthesized. Sucrose density gradients identified a bi-modal MNK distribution with enrichment of MNK in lighter fractions that co-localized with syntaxin 6, a trans-Golgi network (TGN) marker, and in denser fractions containing secretory vesicle markers. Immunostaining and cell surface biotinylation showed MNK redistribution to the plasma membrane with increasing copper levels, while both MNK and DBM redistributed to the plasma membrane following stimulation of exocytosis. Our findings showed a unique MNK localization to the TGN and secretory vesicles in endocrine cells, suggesting distinctive roles for MNK in these compartments and indicating the importance of understanding MNK function in the catecholaminergic system in Menkes patients.