2',3'-Cyclic Nucleotide 3'-Phosphodiesterase: Investigation of Interaction with Fyn Tyrosine Kinase During the Development of Nervous System, and Mitochondrial Import of CNP2 Isoform

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Abstract

2',3'-Cyclic nucleotide 3'-phosphodiesterase is a protein highly expressed in oligodenrocytes in the central nervous system, and it is believed to be an important regulator of myelination. In this report, two aspects of CNP are investigated, each introduced in their own chapters.

First, a possible interaction of CNP with Fyn tyrosine kinase during myelination is investigated. Fyn is an important factor known to be active during the process of myelination, and CNP contains a number of possible tyrosine phosphorylation sites. Furthermore, their presence in an isolated domain of cell membrane called lipid rafts, further strengthened the possibility of interaction. However, no evidence was found which could support the possibility, neither in new born mouse brain nor in in-vitro experiment using transfected cells expressing Fyn and CNP.

Secondly, the role of CNP2 isoform in mitochondria is investigated. CNP2 is found to be associated with mitochondria, and its 20 amino acid segment at the N-terminus possesses characteristics of a mitochondrial import signal. Furthermore, two known sites of serine phosphorylation in the N terminus of CNP2 show influence on mitochondrial localization of the protein. However, results collectively suggests that CNP2 is not imported into mitochondria, as pulse chase did not show the typical

processing of what was suspected to be the N-terminal import signal sequence. Furthermore, CNP was degraded when partially purified mitochondria was subjected to protease action, showing that CNP is not enclosed by mitochondrial membranes. Two serine residues at positions 9 and 22 in the N-terminus of CNP2 are likely phopsphorylated by both PKA and PKC, and is responsible for the decrease in mitochondrial localization of CNP2.

Sommaire

L'enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) est une protéine exprimée par les oligodendocytes dans le système nerveux central. Cette protéine est soupçonnée d'être impliqué dans l'expansion des membranes durant le processus de myélinisation. Au cours de cette étude, deux aspects différents concernant la fonction de CNP ont été évaluées. Les résultats obtenus sont présentés dans deux chapitres distincts.

La première partie de mon travail de recherche consistait à démontrer la possibilité d'une interaction entre CNP et la protéine Fyn lors de la formation de la myéline. Il a été démontré que Fyn était un facteur déterminant nécessaire à la myélinisation des axones. Puisque Fyn possède l'activité tyrosine-kinase, il est donc possible que CNP, qui contient plusieurs résidus tyrosine, soit phosphorylée par Fyn. De plus, CNP et Fyn sont localisées dans des domaines membranaires spécialisés appelés "lipid raft". Malgré toutes ces indications supportant l'hypothèse que CNP et Fyn puissent interagir, nos travaux n'ont démontré aucun lien directe entre ces protéines dans les oligodendrocytes en culture, ou dans des cellules transfectées exprimant simultanément CNP et Fyn.

La seconde partie de mon travail consistait à caractériser la fonction de l'isoforme CNP2. Il a été démontré que la partie N-terminale spécifique à CNP2 possède toutes les caractéristiques d'une protéine pouvant être dirigée vers les mitochondries et que CNP2 est effectivement associée aux mitochondries. Toutefois, nos travaux indiquent que CNP2 n'est pas localisée à l'intérieur des mitochondries. Contrairement à d'autres protéines importées dans les mitochondries, nous avons démontré par des expériences de "pulse-chase" que la partie N-terminale contenant le signal de reconnaissance n'est pas clivée et que la protéine demeure entière. De plus, CNP2 peut facilement être dégradée par l'action de protéases sur des mitochondries intactes, démontrant que CNP2 n'est pas internalisée et protégée de l'action protéolytique. Nous avons également démontré que l'association de CNP2 avec les mitochondries dépend de l'état de phosphorylation de deux résidus sérine présents dans la partie N-terminale de CNP2. Ces résidus sérines en poisition 9 et 22 peuvent être modifiés à la fois par PKC et PKA, et la phosphorylation de ces résidus diminue l'affinité de CNP2 pour les mitochondries.

1. Introduction

2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP) is a peculiar protein possessing a catalytic activity of hydrolyzing 2', 3'-cyclic nucleotides to 2'- nucleotides. As rare as the enzymatic activity, the substrates for such catalysis are yet to be found in the natural environment of CNP. Its function remains mostly unknown even after 40 years since discovery. Lack of natural substrates obscures the relevance of enzymatic activity for its physiological role. However, in recent years, discoveries have progressed and a preliminary sketch of CNP function is being established. CNP is found in the highest amount in myelin, produced by myelinating oligodendrocytes of the central nervous system. Extensive association with cytoskeletal elements suggests involvement in myelination. The presence of CNP in other non-neural tissues also indicates that this protein may have other roles, yet to be identified. This thesis mainly focuses on the possibility of CNP signaling pathways involving Fyn tyrosine kinase during the developmental stages of nervous system, and subcellular localization of CNP2 isoform to mitochondria.

1.1 CNP gene structure and expression

CNP has been identified in many species and their sequences are well established. Comparison of the sequences reveal a relatively high conservation among

the mammals and a significant variation with lower vertebrates. For example, homology of human and mouse CNP is approximately 85% , whereas that of human and bull frog is only 54% (Kasama-Yoshida et. al., 1997). Only a single functional gene is detected in the mouse genome on chromosome 11. The human and mouse CNP genes are organized into 4 exons and two promotors, in the total length of 6~9kB. Two AUG start codons exist in the first and second exons, both possessing separate promoter regions. This results in transcription of two CNP messages responsible for two isoforms. In particular, the shorter CNP transcript coding for CNP2 isoform possesses two AUG start codons in frame, allowing translation of both proteins from a single mRNA (O'Neill et. al., 1997). The resulting two isoforms are identical in their amino acid sequence, with the exception of the 20 amino acids at the N-terminus unique to CNP2. This pattern of protein expression is known as 'leaky scanning', where the initial promoter is not registered by the transcriptional machinery due to sub-optimal conditions of promoter sequence (Kozak, 1995; Kozak 1997). Occurrence of leaky scanning is first observed in rat thymus, where both isoforms of CNP were detected despite the presence of only the longer CNP mRNA (Bernier et. al., 1987). This was explained by O'Neill et. al. (1997) where in vitro translation of synthetic CNP2 mRNA and expression of CNP2 transcripts in cultured cell lines resulted in both protein

isoforms. Futhermore, removal of the second AUG start codon by site directed mutagenesis eliminated the expression of the shorter isoform. It was presumed, from the exclusivity of a shorter mRNA in the central nervous system, that the CNP1 isoform is isolated only in the myelinating oligodendrocytes. However, this finding suggests that both isoforms may be widely expressed in various tissues throughout the body.

Expression of CNP shows tissue specificity which alters through developmental stages. The highest level of expression is seen in myelin in the central nervous system, where CNP comprises approximately 4% of the total protein content. First appearance of CNP is detected prior to myelination, before expression of other well known myelin proteins such as Myelin Basic Protein (MBP) and Proteolipid Protein (PLP) (Sprinkle et. al.., 1978; Snyder et. al., 1983). During embryonic stages, only CNP2 mRNA is detected in the oligodendrocytes, and CNP 1 mRNA is expressed from postnatal day 1. Both messages increase in expression in parallel to expression levels of MBP and PLP, which are up-regulated during myelination. Expression of CNP peaks at postnatal day 10 when oligodendrocyte precursors enter the terminal stage of differentiation preceding myelination (Pfeiffer et. al., 1981; Bansal and Pfeiffer, 1985; Amur-Umarjee et. al., 1990).

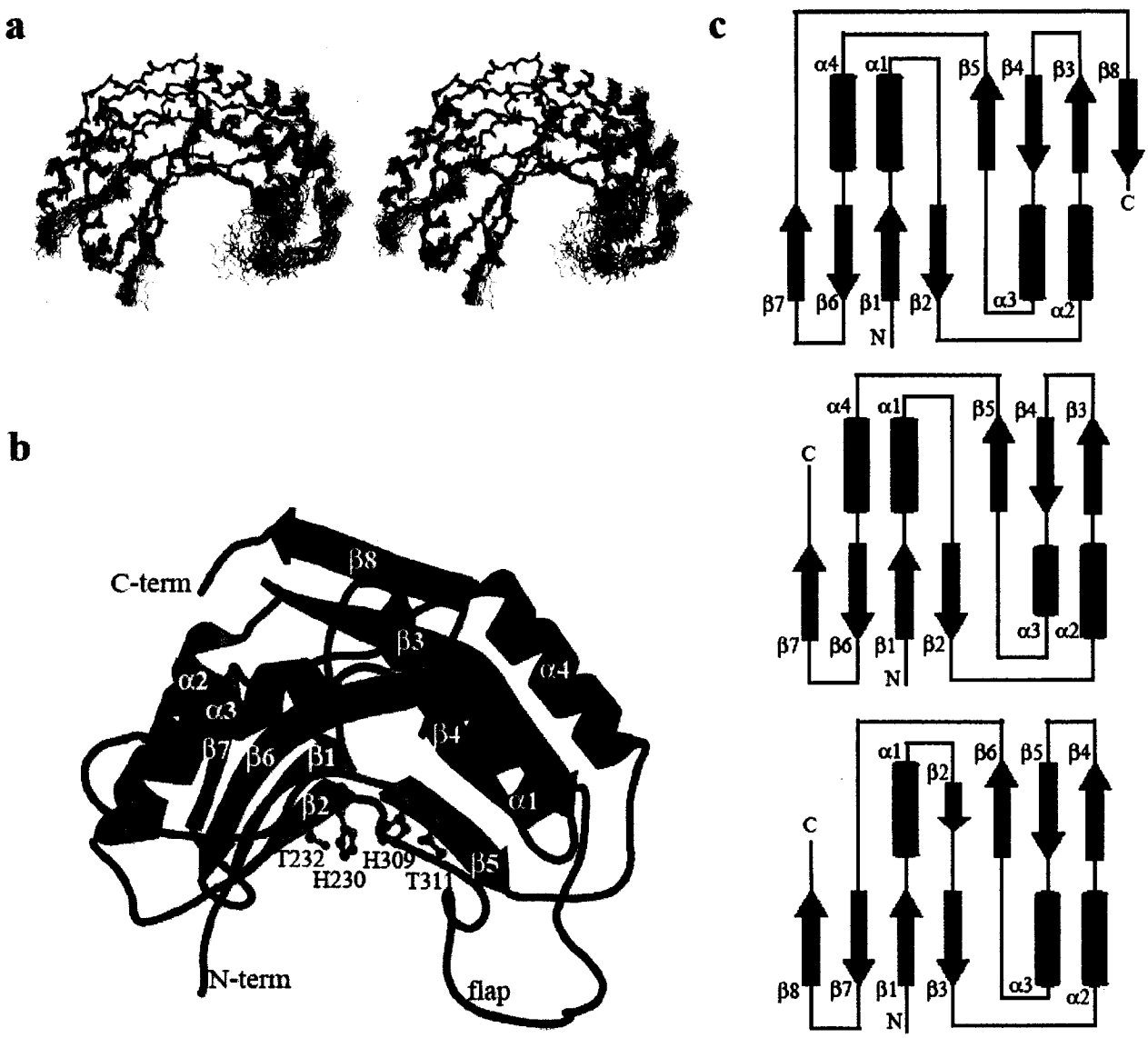
CNP also exists outside of the central nervous system, although expression is limited in quantity compared to that of oligodendrocytes. Expression of CNP is found in various tissues such as myelinating schwann cells of the peripheral nervous system (Edwards et. al., 1988; Stahl et. al., 1990), photoreceptor cells of the retina (Giulian and Moore, 1980), lymphocytes (Weissbarth et. al., 1981), thymus (Bernier et. al., 1987), and adrenal medullary chromaffin cells (McFerran and Burgoyne, 1997). In non-neuronal tissues, CNP2 mRNA is exclusively expressed. Presence of CNP2 mRNA in non-myelinating cells, such as oligodendrocyte precursors and non-neuronal tissues, suggests an additional function of CNP unrelated to the event of myelination.

1.2 Structure of CNP, enzymatic activity and other related proteins

CNP possesses a cyclic phosphodiesterase activity which cleaves the 3' bond of 2', 3' cyclic nucleotides. The enzymatic activity shows strict specificity for 2', 3' cyclic nucleotides, which are not found in the nucleotide pool of cells. However, it does not show significant nucleoside base specificities. Consequently, enzymatic assay of CNP can utilize processing of a synthetic substrate, 2', 3' NADP (Sogin, 1976). Another protein with identical enzymatic activity and highly related structure is Regeneration induced CNP homolog (RICH) found in the regenerating optic nerve of

goldfish. With a high degree of homology in the catalytic regions, these two proteins are found to share similar kinetic parameters for catalysis. Homology of the two protein is mostly found in the C-terminal two-thirds. CNP possesses additional motifs commonly found in many nucleotide binding proteins and enzymes, such as the ATPase "A Box" (G/AXXXXGKT/S) and "B box" (XXXXD, X=hydrophobic residues), as well as the adenine recognition motif (YFGKRPPG). Absence of these functional motifs in RICH suggests that CNP and RICH may have different physiological functions, or that CNP may have additional functions to that of RICH.

The three-dimensional structures of the catalytic fragment of CNP and RICH, determined from NMR studies (Kozlov et. al., 2003), reveal a high structural similarity to several phosphodiesterases which in fact do not have any resemblance to CNP in terms of amino acid sequence (figure 1-1). In particular, CNP has a remarkable resemblance to the plant cyclic nucleotide phosphodiesterase (CPD) from Arabidopsis thaliana, an enzyme involved in the tRNA splicing pathway. Comparing the two proteins, it is evident that both possess the catalytically important two hisitidine and two threonine residues in similar orientation. Therefore, CNP can be structurally classified into a superfamily of proteins involved in RNA metabolism, which include the aforementioned CPD. Thus, RNA is a possible substrate of CNP, and preliminary


a
b
c
C-term
N-term
flap
β8
β3
α4
α2
α3
β7
β6
β1
β4
α1
β2
β5
T232
H230
H309
T311
α4
α1
β5
β4
β3
β8
C
β7
β6
β1
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α3
α2
N
α1
β2
β6
β5
β4
C
β8
β7
β1
β3
α3
α2
N

Figure 1-1: structure of CNP determined by NMR (from Koslov et. al., 2003)

a: backbone superposition of the 20 lowest energy structure

b: ribbon representation of CNP

c: topology comparison of CNP (upper), *A. Thaliana* CPDase (middle), and *T. thermophilus* RNA ligase (lower) from the H2 phosphodiesterase superfamily.

studies of RNA binding show that CNP indeed is able to interact with RNA substrates (Gravel, unpublished data). However, members of this family of proteins are not well understood in terms of their physiological function.

1.3 Post translational modification

Several post translational modifications of CNP are known. They include isoprenylation, palmitoylation and phosphorylation.

A. Isoprenylation

Isoprenlyation allows a close interaction of the modified protein with membranes. Common functions assigned to isoprenylated proteins include effect on cytoskeleton (Hall, 1994; Kranenburg et. al., 1997; Tapon and Hall, 1997), and signal transduction (Casey, 1995; Chou and blenis, 1996; Higgins and Casey, 1996). Analysis of the CNP primary sequence reveals a site of consensus isoprenyl sequence (Gravel et. al., 1994). Isoprenylation of CNP occurs towards the C-terminus (Braun et. al., 1991), which initiates further modification by removal of the last three amino acid residues and methylation of the carboxyl group (Clarke, 1992; Cox et. al., 1994). Functionally, isoprenylation of CNP plays an important part, as modified CNP lacking the isoprenylated cysteine fails to induce morphological changes when transfected (De

Angelis and Braun, 1994). Possibility of CNP in a signal transduction is suggested by the finding of CNP in association with lipid rafts, a membrane subdomain which plays an important role in signaling.

B. Phosphoryation

Phosphorylation is one of the most wide spread mechanisms by which intracellular events are regulated. Due to the wide spread use, functions under the control of phosphorylation includes nearly all aspects of cellular activity, starting from simple activation and deactivation of a single enzyme to complex signaling cascades leading to apoptosis. Both CNP1 and CNP2 are modified by phosphorylation. CNP2 is found to be the more heavily phosphorylated isoform, suggesting CNP2 may possess additional phosphorylation sites located in the N-terminal sequence. Unique phosphorylation patterns of CNP2 suggests that phosphorylation may be a signal which distinguishes functions of the two isoforms. CNP phosphorylation seems to be stimulated by activators of protein kinase A (PKA) and protein kinase C (PKC) (Bradbury and Thompson, 1984; Agrawal et. al., 1990; Stricker et. al., 1994). Two serine residues found in the N-terminus of CNP 2 are the only targets of phosphorylation identified thus far (O'Neill and Braun, 2000). Phosphorylation of these two residues have been found to regulate localization of CNP2 to mitochondria.

Control of subcellular localization by phosphorylation is not a rarity, as seen in mitochondrial localizations of apoptotic Bad (Bertolotto et. al., 2000; Harada et. al., 1999; Zha et. al., 1996) and Glutathione S-Transferase A4-4 (Robin et. al., 2003) , and nuclear proteins such as thyroid hormone receptor Alpha1 (Nicoll et. al., 2003). Furthermore, phosphorylation of localization signal sequence itself is often observed in regulating the destination of the protein (Reimer et. al., 2003, Robin et. al., 2003; Valovka et. al., 2003).

Tyrosine phosphorylation of CNP is a possibility as well. Amino acid sequence of CNP indicates that CNP contains possible targets of phosphorylation by Src family of kinases. It is interesting to note that Fyn tyrosine kinase, a member of Src kinase family, is active during myelination (Umemori et. al., 1994). Fyn is found to coimmnoprecipitate with CNP (personal communication from Dr. Osterhout) and both proteins are found to localize to lipid rafts, a subdomain of plasma membrane enriched with sphingolipids and signaling molecules.

1.4 Functional relevance of CNP

The function of CNP still remains largely obscure, but there are findings which may be helpful in understanding its physiological role. Expression of CNP in HeLa

cells results in dramatic morphological transformations with numerous outgrowth of filopodia, suggesting association/interaction with cytoskeleton. With elimination of the isoprenoid tail, this transformation disappears, suggesting CNP may be an anchor in the membrane which associates with cytoskeletal elements (DeAngelis and Braun, 1994). Indeed, CNP is found to interact with two major cytoskeletal proteins, tubulin and actin. Co-localization of CNP and tubulin is shown in cultured primary and differentiated oligodendrocytes (Dyer and Benjamins 1989). CNP also possesses microtubule polymerization activity in vitro (Bifulco et. al., 2002). Furthermore, co-immunoprecipitation of CNP identified tubulin as a major binding partner by mass spectrometry (Lee, unpublished data). Association of CNP with actin is also observed (De Angelis and Braun, 1996).

Transgenic knockout mice have been created in an effort to study the function of CNP relating to myelination. Surprisingly, myelin in these mice shows little abnormalities (Lappe-Siefke et. al, 2003). Oligodendrocytes seem to function normally and myelination commences without any defects. However, when the transgenic mice reach adulthood, a severe neurodegenerative disorder is observed. These mice have axonal swellings and Wallerian degeneration causing a large microglial response manifested as hydrocephalus and premature cell death. Results

indicate that the function of CNP may not be limited to the stage of myelination, and plays an essential role in the maintenance of normal neuronal integrity after completion of development.

Transgenic mice overexpressing CNP were created by introduction of the human gene, resulting in increase CNP expression by 6 folds (Gravel et. al., 1996). Major changes observed in these transgenic mice are presence of large vacuoles surrounded by myelin membranes that extended from myelin internodes, and failure of the membrane fusion leading to loss of major dense lines. Furthermore, investigation of these mice early in development revealed aberrant oligodendrocyte and myelin membrane formation during early stages of oligodendrocyte differentiation. Overexpression of CNP is suspected to interfere with the function of MBP, causing defective myelin structure.

Lastly, structure of CNP discovered by NMR indicates that CNP may be an RNA binding/processing molecule. Structural similarities with tRNA ligase and plant cyclic phosphodiesterase suggests CNP is highly related structurally to these proteins, raising possibility of CNP functioning as an RNA modifying enzyme. Also, the catalytic domain possesses motifs which are found in RNA binding molecules from

fungus and bacteria. Furthermore, binding of CNP to some RNA virus and viral nucleocapsid strengthens such a possibility (Rosenbergova et. al., 1991).

1.5 Mitochondrial import

With the exception of a few proteins encoded by the mitochondrial genome, most proteins enter mitochondria through an orchestrated transport system of outer and inner mitochondrial membranes. Many proteins which are transported into mitochondria have an N-terminal segment, called mitochondrial matrix targeting signal (MTS), that serves as a signal for the import into mitochondria. Recognition of the import signal by the TOM (Translocase of the Outer Membrane) complex initiates the process. TOM complex forms a pore through which proteins are selectively transported upon the recognition of the signal sequence. Once in the intermembrane space, the protein is recognized by the TIM (Translocase of the Inner Membrane). The process of mitochondrial import is a highly coordinated event involving several chaperons to maintain the expressed protein in an import-competent state. An example is heat shock protein 70 (HSP70), which is found in the matrix of mitochondria. Hydrolysis of ATP is required for the proper functioning of the import. Also essential

for the process of import is the electrical membrane potential across the inner membrane. After proteins are brought into the matrix, the signal sequence is usually cleaved by mitochondrial matrix peptidases.

1.6 CNP2 isoform: a brief overview

CNP2 differs from CNP1 in its N-terminal 20 amino acid sequence. The function of the additional sequence has been obscure for many years, and recently it was discovered that CNP2 isoform is localized to mitochondria. There has been a report of mitochondrial localization of CNP in cultured adrenal medulla chromaffin cells (McFerran et. al., 1997), although it was not specified which isoform is responsible for the localization. Investigation of the N-terminal sequence of CNP2 suggests it is solely responsible for targeting of CNP to mitochondria. First, CNP1, which lacks the N-terminal sequence, is not observed to be localized to mitochondria. Secondly, synthetic fusion protein made up of the N-terminal sequence of CNP2 and Green Fluorescent protein (GFP) localizes to mitochondria. Thirdly, CNP co-fractionates with mitochondria in subcellular fractionation. There is a possibility that the N-terminal 20 amino acid sequence of CNP2 is a mitochondrial import signal (MTS). Analysis of the N-terminal extension shows that it is well conserved in different species, and it

possesses a net positive charge, no acidic residues, and some hydroxylated and hydrophobic residues. Secondary structure prediction of the region indicates it shows the amphipathic nature of the helix, which is a common trait of the MTS (Neupert, 1997; von Heijne, 1986; von Heijne et. al., 1989). Analysis of the sequence suggests that a cleavage site is located between the 20^{th} and 21^{st} residues of the CNP2, which would yield a protein identical to CNP1 in the mitochondrial matrix.

Expression of CNP2 isoform is usually contaminated with CNP1, due to the aforementioned alternative initiation. However, by elimination of the second AUG start codon, it is possible to express the CNP2 isoform exclusively. Substitution of methionine 21 with leucine (M21L) results in dramatically reduced production of CNP1, which provides a useful tool for the study of CNP2 isoform (O'Neill and Braun, 2000). Expression of this modified protein in HeLa S3 results in CNP2 predominantly localized to mitochondria. Approximately 85% of transfected cells show mitochondrial localization of CNP.

A feature of interest in the N-terminal sequence of CNP2 is presence of several serine residues. Of the two isoforms, CNP2 is found to be more heavily phosphorylated. Hence, the serine residues found in the N-terminal sequence of CNP2

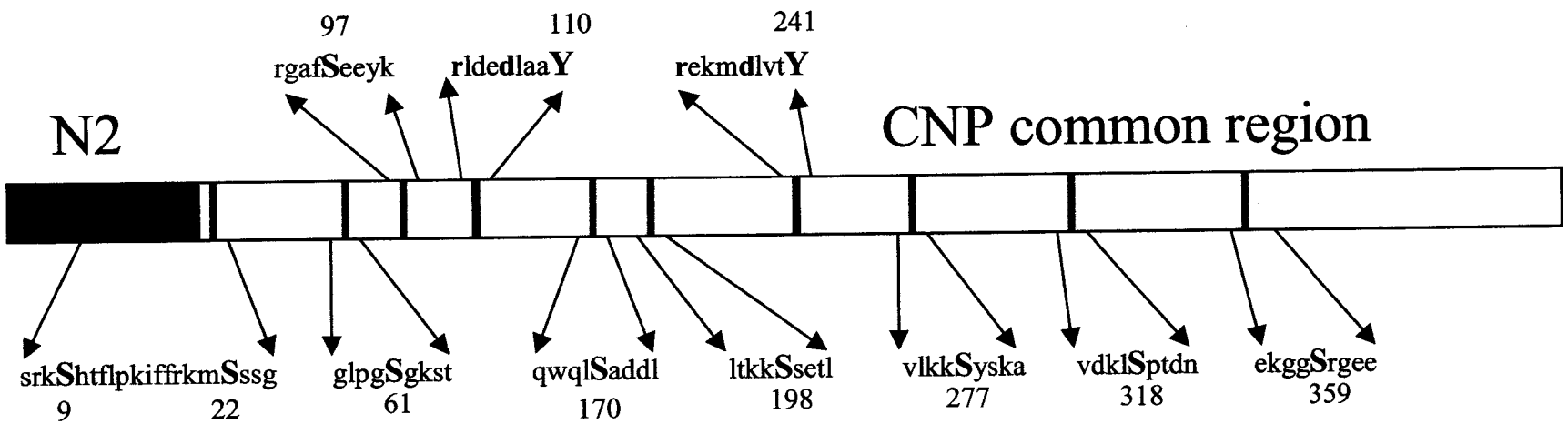
are suspected to be targets of phosphorylation. Tryptic phosphopeptide mapping of CNP2 suggests that two residues of CNP2 are phosphorylated (O'Neill and Braun, 2000). Likely candidates are serine 9 and serine 22 since they are both contained in possible consensus PKA/PKC phosphorylation motifs. Phosphorylation of CNP2 may be involved in the regulation of mitochondrial localization. It is found that elimination of phosphorylation by substitution of serine 9 and serine 22 to alanines results in enhanced mitochondrial localization. On the other hand, when the serines are exchanged with aspartic acids, which mimics phosphate group with the negative charge of the carboxylic group, CNP2 loses its ability to localize to mitochondria (O'Neill, Ph. D thesis, 2001).

Chapter 2: phosphorylation of CNP by Fyn tyrosine kinase

2.1 Introduction

Numerous reports have shown that CNP is phosphorylated on several different residues. Previous studies reported presence of several serine residues in CNP phosphorylated by phorbol-ester sensitive kinase (Agrawal et. al., 1990, 1994) and cyclic-AMP dependent kinase (Bradbury and Thompson, 1984; Stricker et. al, 1994). Amino acid sequence of CNP indicates numerous possible sites of phosphorylation by serine/threonine kinases such as PKA, PKC, Casein kinase II, as well as two or more conserved tyrosine residues possessing consensus phosphorylation motifs [(R/K)-X (2,3)- (D/E)-X(2,3)-Y] (figure 2.1). Thus far, two serine residues (serine 9 and 22) within the N-terminal sequence of CNP2 have been identified as targets of phosphorylation, and are speculated to play a role in the regulation of localization to mitochondria (O'Neill and Braun. 2000). One additional residue, likely in the common region of CNP1 and CNP2 isoforms, is found to be phosphorylated, but remains unidentified. This results in a heavier phosphorylation of CNP2 isoform in comparison to CNP1.

Speculations of tyrosine phosphorylation of CNP was initiated by observations of Dr. Donna Osterhout. According to her report, Fyn tyrosine kinase was found to be co-immunoprecipitated with CNP from mature rat oligodendrocytes. Also, CNP was



— possible phosphoserine residue

— possible phosphotyrosine residue

Figure 2-1: CNP contains numerous target sequences of various serine/threonine and tyrosine kinases. Candidate kinases of CNP phosphorylation include PKA, PKC, casein kinases 1 and 2. Due to phosphorylation of serine 9 and 22, CNP2 is more heavily phosphorylated than CNP1. CNP1 is phosphorylated as well, but the target residue(s) remain(s) unknown. Amino acid sequence of CNP is analysed by Expasy post translational modification prediction program at http://ca.expasy.org/cgi-bin/scanprosite.

recognized by phosphotyrosine antibodies, which are able to identify single tyrosine residues modified with phosphate groups. Furthermore, there was an indication of a 110kDa protein complex composed of CNP and Fyn, which apparently remains intact through the denaturing conditions of SDS-PAGE. Dr. Osterhout's findings are further supported by the recent reports of localization of CNP and Fyn to a microdomain of lipid membrane bilayer called lipid rafts of DIGs (detergent-insoluble glycosphingolipid-rich microdomains) (Kim and Pfeiffer, 1999; Kramer et. al., 1999).

Fyn is a member of the Src family, which includes 8 other tyrosine kinases such as Src, Lyn, Yes and Lck. General functions attributed to Src family kinases are cell adhesion, cytoskeletal rearrangements, cell migration, regulation of the cell cycle, apoptosis, and differentiation (Boschek et. al., 1981; Hecker et. al., 1991; Twamley-Stein et. al., 1993; Kaplan et. al., 1995; Rodier et. al., 1995; Huang et. al., 1997). Fyn, in particular, is shown to play various roles in the process of myelination. Despite the presence of several members of Src kinase family in the brain during the developmental stages, Fyn is most noticeable in terms of expression level and catalytic activity (Umemori et. al., 1992, Bare eat al., 1993). Fyn activity reaches a peak during the early stages of myelination (Umemori et. al., 1994), and inhibition of Fyn results in reduced process extension and myelin membrane formation (Osterhout et. al., 1999).

Furthermore, Fyn stimulates the expression of MBP, a major component of myelin responsible for its structural integrity (Umemori et. al., 1994, 1999), and also interacts with cytoskeletal elements such as Tau and other signaling molecules such as RhoGAP leading to the morphological changes (Wolf et. al., 2001). Absence of Fyn in cultured oligodendrocytes from Fyn knock-out mice results in reduced number of cells reaching morphological maturity, and also in failure to respond to a morphological maturation stimulus such as insulin-like growth factor-I (Sperber et. al., 2001). Considering the involvement of Fyn and CNP in the process of myelination, report of CNP-fyn interaction by Dr. Osterhout, and presence of unknown phosphorylated residue(s) in CNP, tyrosine phosphorylation of CNP by Fyn was an intriguing possibility.

To investigate the interaction of Fyn and CNP and tyrosine phosphorylation of CNP, mouse brains of different ages were taken and examined for the presence of phosphorylated tyrosine residues on CNP, and possible interaction of CNP and Fyn. Same experiment was carried out using cultured cells expressing CNP and Fyn.

2.2 Materials and methods

1. Mouse brain homogenization and immunoprecipitation of CNP

Mouse brains (2 to 6 weeks old) were homogenized with a Dounce homogenizer in ice cold RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 5mM EDTA, 1% NP40, 0.5% Na Deoxycholate, 0.1% SDS, and 1mM Na_3VO_4, with aprotinin, Leupeptin, peptistatin and Phenylmethanesulfonyl fluoride added before each use) with 20 strokes. The homogenate was centrifuged in a microcentrifuge for 30 minutes at 13,000RPM in 4°C. The supernatant was precleared with protein G sepharose (pharmacia Biotech) (100µl per1ml of homogenate) for 1 to 2 hours to eliminate non-specific binding. The precleared supernatant was then mixed with protein G sepharose beads cross-linked with mouse monoclonal CNP antibody (Sternberger Monoclonals Inc.), and incubated overnight at 4°C. Beads were washed 5 times in RIPA, and once more in 1/10 dilution of RIPA. Protein was eluted with 150µl of low pH buffer (50mM glycine pH 2.5 and 0.1% Triton X-100) at room temperature for 5 minutes. The pH of eluate was neutralized by the addition of 30µl of 1M Tris pH 8.0 and then concentrated by methanol precipitation as follows. 4 volumes of methanol and 1 volume of chloroform were added and mixed by vortexing. After 15 minutes of incubation on ice the mixture was centrifuged down in a microcentrifuge at 13000RPM for 15 minutes at 4°C.

The top layer was carefully removed, adding 3 more volumes of methanol to the remainder. The mixture was centrifuged for 5 minutes and resulting protein pellet is dried and solublized in SDS sample buffer.

Antibodies were cross linked to protein G sepharose using the following protocol. 50µl of beads were washed in PBS+ 0.1% Triton X-100 (TX). 10µl of antibody was mixed with the beads in 1ml of PBS+0.1% TX and incubated for 4 hours at 4°C. The beads were washed 4 times with PBS+ 0.1% TX and 3 times with 0.2M Sodium Borate pH 8.6. Further incubation of beads was done in 100µl of buffer containing 20mM DMP and 0.2mM triethanolamine pH 8.3 for half an hour at room temperature. Beads were subsequently quenched in 1ml of 0.2M ethanolamine HCl pH 8.2 for 5 minutes at room temperature, and incubated in same buffer for 1 hour at room temperature. Resulting beads were washed twice in PBS+ 0.1% TX and incubated in 1ml of 50mM glycine HCl pH 2.5 for 5 minutes at room temperature. Finally, the beads were washed in 1ml of PBS+0.1% TX 3 times and used for immunoprecipitation.

2. Co-expression of Fyn and CNP in cultured cells

PcDNA-Fyn expression vector was generated by subcloning the Fyn cDNA (previously made in our lab) into the BamHI and EcoRI sites of pcDNA empty vector. rcCMV-CNP1 was made by O'Neill (Ph. D thesis, 2001).

PcDNA Fyn and RcCMV CNP1 were transiently cotransfected into COS 7 cells at 60% confluency using FuGENE (Roche) according to the manufacturer's protocol. Cells were incubated 24h to 48hrs to allow efficient expression of transfected genes. Cells were washed in PBS twice, and lysed with 1ml of RIPA per 100mm dish. After 5 minute incubation on ice, cells were scraped using rubber cell scraper, and passed through a 16G needle 10 times to further facilitate lysis. The resulting cell lysate was centrifuged down in a microcentrifuge for 30 minutes at 13000RPM at 4°C. Immunoprecipitation of CNP was carried out in the same manner as mentioned above.

3. SDS-PAGE and Western blot

Immunoprecipitated protein samples in SDS-PAGE sample buffer were heated at 90°C for 5 minutes prior to separation on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto nitrocellulose

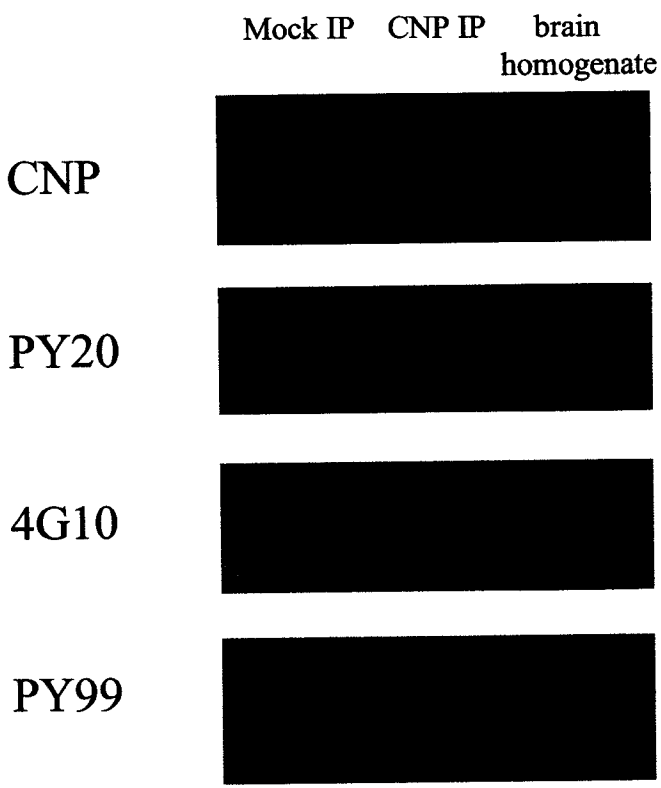
membranes. After the completion of transfer, membranes were air dried for 5~10 minutes and blocked for 1 hour in TBST (10mM Tris-HCl pH 8, 150mM NaCl, and 0.05% Tween 20) containing fatty acid-free 5% bovine serum albumin (BSA) for phosphotyrosine blots, or 5% skim milk in TBST for CNP blots. Primary antibody was incubated with membranes overnight in 4°C on a shaker. Membranes were washed in TBST 3x10 minutes each. Following the washes, membranes were incubated with secondary antibody for 1 to 3 hours, and washed in TBST 3x10 minutes. Enhanced Chemiluminescence (NEN Perkin Elmer Life sciences Inc.) was used to detect the protein of interest according to the manufacturer's protocol. Primary antibodies used were diluted as follows: PY-20 mouse α-phosphotyrosine antibody (1:3000 to 1:6000), 4G10 mouse α-phosphotyrosine antibody (1:3000), PY99 mouse α-phosphotyrosine antibody (1:1000), were diluted in TBST +5% fatty acid-free BSA, and α-CNP mouse monoclonal antibody (1:10,000 to 1:30,000) was diluted in TBST +1% skim milk powder. Horse radish peroxidase conjugated goat α-mouse antibody was used as the secondary antibody (1:10,000), in TBST + fatty acid free 5% BSA for phosphotyrosine blots or in TBST +1% skim milk powder for CNP blots.

2.3 Results

1. CNP from Mouse brains of various developmental stages is not phosphorylated on tyrosine residues

Expression of Fyn is detected early in embryonic stages (E18) of rat brain (Bare et. al., 1993), and its activity peaks at the most active stage of myelination (Umemori et. al., 1994). Therefore it was necessary to test tyrosine phosphorylation of CNP taken from mouse brains of different ages to address the right stage of development. Two week and six week old mice were chosen as they represent active development and myelination, and completion of developmental stage. Detection of phosphotyrosine is enabled by use of antibodies which specifically recognize individual tyrosine residues modified by phosphorylation. Specificities of α-phosphotyrosine antibodies vary from one to another, therefore it was necessary to test CNP with different antibodies to ensure detection of any possible phosphotyrosine residues. All antibodies recognized several bands in the adult mouse brain homogenate (6 week old), indicating that catalytic activity of tyrosine kinase is present (figure 2.2A). In comparison to adult brains, 2-week old littermate brain homogenates showed more phosphorylated proteins in higher intensities, showing that catalytic activity of tyrosine kinase is upregulated at this stage of development (figure 2.2B). However, all

A


Mock IP
CNP IP
brain homogenate
CNP
PY20
4G10
PY99

B

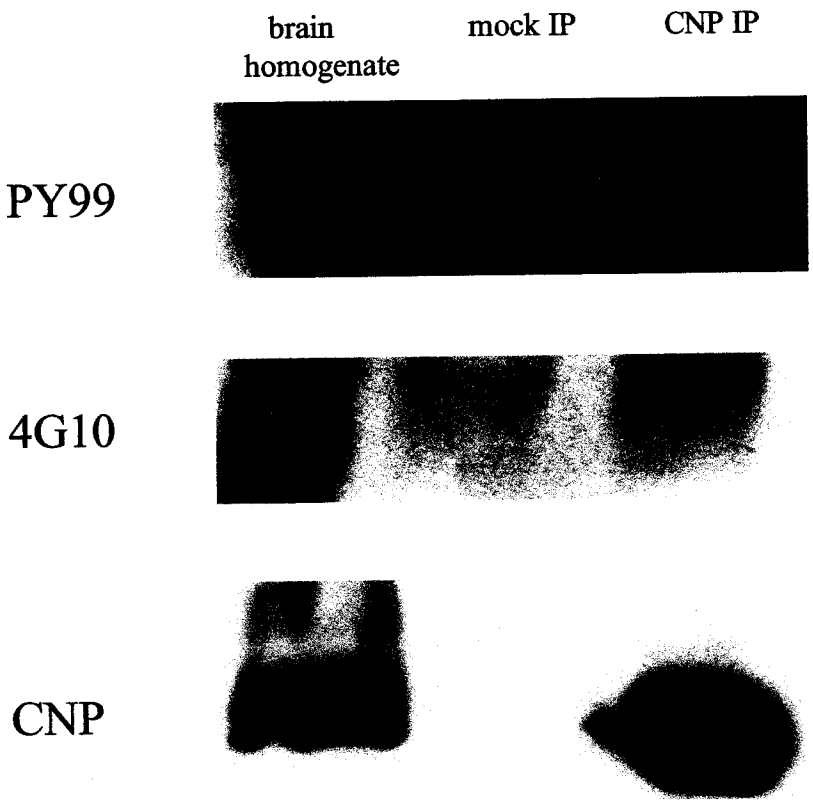

brain homogenate
mock IP
CNP IP
PY99
4G10
CNP

Figure 2-2: CNP from two and six week old mouse brains did not show any sign of tyrosine phosphorylation.

A: Brains from 6 week old mice were taken and immunprecipitated using α-CNP mouse antibody. Tyrosine kinase activity was present as several protein bands were detected using α-phosphotyrosine antibodies (PY20, 4G10, PY99). Immunoprecipitates contain CNP, but show no signs of tyrosine phosphorylation.

B: 2 week old mouse brains were tested for tyrosine phosphorylation of CNP in the same manner as above. Heavier tyrosine kinase is observed in the brain homogenate in comparison to 6 week old mice. Immunoprecipitate contained large amount of CNP, which failed to be recognized by phosphotyrosine antibodies.

α-phosphotyrosine antibodies failed to recognize any phosphotyrosine in CNP immunoprecipitates (figure 2.2 CNP IP).

2. Co-expression of CNP and Fyn in COS 7 cells do not result in tyrosine phosphorylation of CNP

Transfection of COS 7 cells with CNP and Fyn resulted in expression of both proteins in high quantities easily detectable by western blot (figure 2-3 CNP and Fyn). Activity of Fyn is apparent from the fact that Fyn itself is heavily tyrosine-phosphorylated (figure 2.3 P-Tyr, Fyn alone, and CNP and Fyn). Despite the presence of Fyn and its catalytic activity, CNP did not show any signs of interaction. Co-expression of CNP and fyn did not result in tyrosine phosphorylation of CNP as all the α-phosphotyrosine antibodies did not recognize CNP (Figure 2.3 P-Tyr). Furthermore, immunoprecipitation of CNP did not result in co-immunoprecipitation of Fyn (figure 2.3 Fyn).

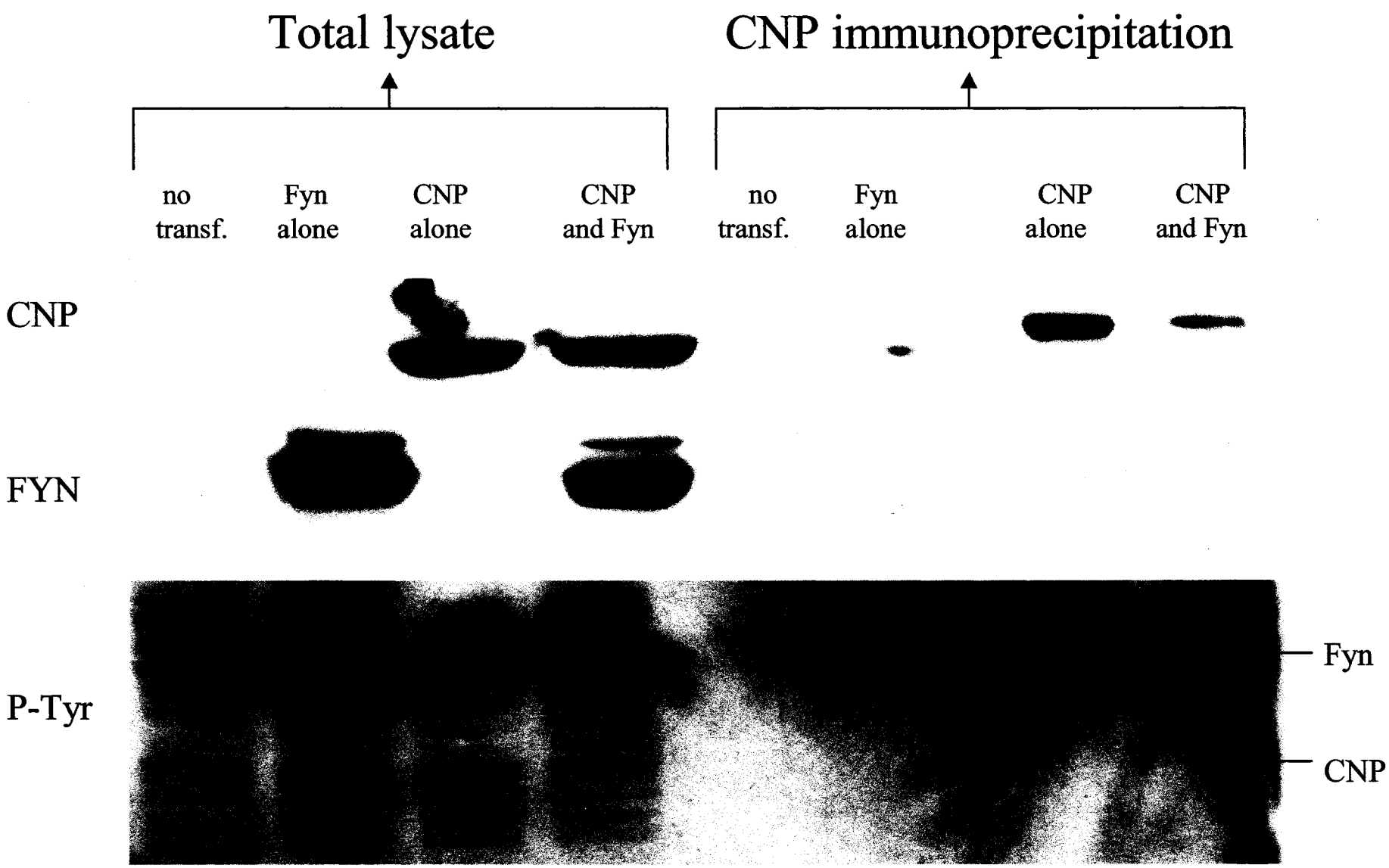

Total lysate
CNP immunoprecipitation
no transf.
Fyn alone
CNP alone
CNP and Fyn
no transf.
Fyn alone
CNP alone
CNP and Fyn
CNP
FYN
P-Tyr
Fyn
CNP

Figure 2-3: Transfected COS-7 cells show no interaction of CNP and Fyn.

COS-7 cells were transiently transfected with PcDNA-Fyn and RcCMV-CNP1, individually or simultaneously. Cell lysates were immunoprecipitated using α-CNP mouse antibody cross-linked to protein G-sepharose. Both cell lysates and immunoprecipitates are analyzed by western blot using Fyn, CNP and phosphotyrosine antibodies. Transfection resulted in good expression of Fyn and CNP. Despite presence of both proteins in COS-7, CNP does not react to phosphotyrosine antibodies. (lane 'CNP and Fyn', probed with P-Tyr). Additionally, CNP immunoprecipitation failed to bring down Fyn, indicating there is no direct interaction between the two proteins (CNP immunoprecipitation, lane 'CNP and Fyn', probed with Fyn antibody).

2.4 Discussion

Two different approaches were employed to study the possibility of Fyn-CNP interaction. However, they did not yield any results to support this. Mouse brains at different developmental stages (2 and 6 weeks old) were examined for CNP tyrosine phosphorylation. In all cases, although tyrosine phosphorylated proteins were detected in the cell lysate, there was no evidence of CNP tyrosine phosphorylation. All three phosphotyrosine antibodies failed to recognize CNP, despite its clear abundance in the immunoprecipitate. It was initially speculated that CNP may be tyrosine-phosphorylated during the early developmental stages of myelination when Fyn kinase is highly active. However, two-week old littermates did not show any tyrosine phosphorylation of CNP, suggesting that regulation of myelination by Fyn kinase does not involve CNP.

As a second approach to examine the direct interaction of CNP and Fyn, both CNP and Fyn are overexpressed in non-neuronal COS-7 cells by transient transfection. Overexpression of both proteins would ensure the presence of Fyn tyrosine kinase in the vicinity of CNP. However, results did not indicate any hint of CNP phosphorylation by Fyn. Despite the large amount of CNP pulled down by immunoprecipitation

(figure 2.2 and 2.3), various phosphotyrosine antibodies failed to recognize CNP (figure 2-2 P-Tyr). Furthermore, Fyn failed to co-immunoprecipitate with CNP (figure 2-2 Fyn), which is contrary to what has been reported by Dr. Osterhout. Therefore it is highly unlikely that CNP is a target for Fyn tyrosine kinase.

Although there have been numerous reports of serine/threonine phosphorylation of CNP (Agrawal et. al., 1990, 1994; Bradbury and Thompson, 1984; Stricker et. al, 1994; O'Neill et. al, 1997), preliminary data from Dr. Osterhout supported a possibility for tyrosine phosphorylation of CNP. Immunoprecipitation of CNP from cultured oligodendrocyte lysates using α-CNP mouse monoclonal antibody was reported to pull down Fyn, and both CNP and Fyn reacted to α-phosphotyrosine antibodies, suggesting that CNP is a substrate for Fyn tyrosine kinase (figure 2.1). Furthermore, a complex of Fyn and CNP migrated with an apparent molecular weight of 110kDa. However, the data are questionable for the following reasons. First, western blots of CNP immunoprecipitations were stripped and reprobed with different mouse antibodies. Despite the presence of denaturing agents in the stripping solution and high incubation temperature, complete removal of antibodies can be difficult to achieve, which may result in false signals. Second, mouse antibodies were used without cross-linking to

the sepharose beads for the immunoprecipitation. This results in presence of mouse antibody in the immunoprecipitated samples, which shows up as a 45kDa band on SDS-PAGE. This will react with α-mouse secondary antibody, and may be misinterpreted as CNP. Third, it is questionable how the high molecular weight CNP-Fyn complex could endure denaturing conditions of SDS-PAGE. Despite the use of very mild lysis buffer (150mM NaCl, 1% non-ionic detergent NP40) for the immunoprecipitation to preserve native complexes, the interaction of the two protein is likely to be disrupted SDS-PAGE. It is likely that the 100kDa band identified by western blot is a non-specific protein cross-reacting with the CNP antibody.

Our data presented above largely argues against the tyrosine phosphorylation of CNP by Fyn. Interaction of Fyn with cytoskeletal elements have been discovered (Klein et. al., 2002; Lee et. al., 1998), and Fyn is believed to be necessary for the morphological changes leading to oligodendrocyte differentiation (Osterhout et. al., 1999). However, our results suggest such effects are not likely to involve CNP directly.

Chapter 3: mitochondrial association of CNP2

3.1 Introduction

CNP exists in two different isoforms. CNP1 is the predominant form found in the central nervous system, which lacks the N-terminal 20 amino acid sequence (N2) of CNP2. CNP2 transcript is expressed exclusively in embryonic brain and several non-neural tissues, and this mRNA was found to be responsible for translation of both CNP1 and CNP2 isoforms through alternate AUG codon usage (O'Neill et. al., 1997). Aside from the obvious difference in the sequence, CNP2 differs from CNP1 in at least two aspects. First it is not the major form of CNP expressed in the nervous system. CNP1 mRNA is mainly restricted to the nervous system, whereas CNP2 mRNA is more widely expressed in various tissues. Second, it is the more readily phosphorylated isoform of CNP (Agrawal et. al., 1990; Agrawal et. al., 1994; Sprinkle 1989; Vogel and Thompson, 1988). The sequence difference between the two is only the N-terminal 20 amino acid stretch of CNP2, which suggests that the N-terminal sequence is highly phosphorylated.

The function of CNP has not been established clearly. There has been abundant evidence of association with cytoskeleton elements as well as a possible role in signal transduction, suggesting involvement in the event of myelination. CNP2 isoform, on the other hand, is suspected to have a more general role, due to its wide

expression in a variety of tissues. Also the presence of N2 in CNP2 suggests a function that may differ from that of CNP1. These 20 amino acid residues have been shown to possess characteristics of a mitochondrial targeting/import signal with possibility of cleavage after lysine 20 (figure 2-1). The work of a previous lab member showed that CNP2 is indeed targeted to mitochondria, and the fusion protein of the N-terminal sequence and GFP was also found to be associated with mitochondria. Furthermore, mutation of serine residues, which are suggested to be the sites of phosphorylation, results in changes to the mitochondrial targeting of CNP2. The results indicate that phosphorylation is a negative regulator of mitochondrial targeting, as mutation of serine to alanine (non-phosphorylatable residue) results in enhanced localization to mitochondria, whereas mutation of serine to aspartic acid (mimics phosphorylated serine with the charged functional group) almost completely abolishes this localization (O'Neill, PhD thesis 2001).

With the knowledge that CNP2 is targeted to mitochondria, it is important to determine whether localization is due to import into mitochondria, or by association to the mitochondrial outer membrane or the components of the membrane. Also it is important to determine how phosphorylation affects the mitochondrial localization.

3.2 Materials and methods

1. Construction of CNP2 expression vector and its variations

Constructs prepared by O'Neill were used with no modifications (O'Neill et. al., 1997, 2000). All the constructs used in this report are described in the table below.

abbreviation	Details of modification
CNP1	CNP construct excluding the N-terminal 20 amino acids
M21L	CNP2 with mutation of second methionine to leucine. Produces CNP2 isoform exclusively
S9A	M21L construct with mutation of serine 9 to alanine
S22A	M21L construct with mutation of serine 22 to alanine
S9,22A	M21L construct with mutation of serines 9 and 22 to alanines
S9D	M21L construct with mutation of serine 9 to aspartate
S22D	M21L construct with mutation of serine 22 to aspartate
S9,22D	M21L construct with mutation of serines 9 and 22 to aspartates
C417S	M21L construct with mutation of cysteine 417 to serine

Table 1- various DNA construct of CNP used in transfection of mammalian cultured cells.

2. Treatment of cells transfected with various constructs of CNP2 with kinase activators and inhibitors

HeLa S3 cells were plated onto glass coverslips to 50~60% confluency. The next day, cells were transfected with mouse CNP2 cDNA cloned into RcCMV mammalian expression vector with mutation of methionine 21 to leucine (M21L) along with its variations (O'Neill et. al., 1997, table 1), using FuGENE transfection reagent (Sigma-Aldrich) using manufacturer's protocol. After 4~6 hours of transfection, the media was removed and various drugs were administered along with fresh media. Cells were incubated overnight and processed 24 hours after transfection as follows. Cells were fixed in 4% paraformaldehyde in PBS for 20 minutes, and permeablized in 0.3% Triton X-100 in PBS. They were then washed in PBS twice and incubated with 5% BSA in PBS for 30 minutes. Primary antibody was incubated on the coverslips for 1 hour, followed by 3 washes of PBS+0.1% Triton X-100 for 10 minutes. Secondary antibody was incubated for 30 minutes and then washed 3 times with PBS+0.1% Triton X-100 for 5 minutes. They were rinsed in distilled water and mounted on a microscope slide using Immunomount (Fischer Scientific). Antibodies were diluted in 5% BSA in PBS, to following concentrations: α-CNP mouse monoclonal antibody 1/500, α-TOM20 rabbit antibody 1/500, α-mouse antibody Texas Red and FITC 1/200,

goat α-rabbit antibody FITC 1/200, donkey α-rabbit Texas red 1/200. Alternatively, mitochondria were stained using MitoTracker Red CMXRos (Molecular Probes), by incubating cells at 37°C for half an hour at a concentration of 100nM in Dubelcco's modified Eagle's Medium (DMEM), prior to fixation. Drugs were used in following concentrations: PDB, PDD, PMA100~500nM; DbcAMP, SpcAMPs 1~5mM.

3. Pulse and Chase

HeLa S3 cells were transfected with M21L using FuGENE according to the manufacturer's protocol. After 48 hours they were washed twice in PBS and incubated in methionine and cysteine free DMEM without serum for 30 minutes. Cells are labeled using S-35 Express containing radioactive methionine and cysteine (Perkin Elmer) diluted to 0.2mCi in methionine, cysteine-free DMEM for 5~15 minutes. Cells were washed twice in PBS and incubated in regular DMEM containing 10% fetal bovine serum (FBS), 2mM L-glutamine and penicillin/streptomycin (Gibco BRL) until the time of harvest. SDS-free RIPA was used to lyse cells directly from the plates. (1ml /100mm dish) Lysates were scraped together by rubber cell scrapers and collected into microcentrifuge tubes. Lysates were kept frozen until all the samples have been harvested. They were sonicated briefly for 20 seconds to eliminate any

unbroken cells. The resulting lysates were spun down at the top speed using a table top microcentrifuge for 30 minutes, and the supernatant was taken and incubated with 50µl of Protein G sepharose for 3 hours to eliminate non-specific binding to the beads, all at 4°C. After pre-clearing with sepharose beads, the supernatant was mixed with another 50µl of fresh Protein G-sepharose, with the addition of 5~10µl αCNP mouse monoclonal antibody and incubated overnight at 4°C. The mixture was spun down briefly in a table-top microcentrifuge and the supernatant was discarded. The beads were washed 5 times in 1ml RIPA, and once in 1:10 dilution of RIPA. CNP was eluted by incubating the beads in 150µl 50mM glycine pH 2.5 + 0.1% Triton X-100, for 5 minutes on a shaker at room temperature. The mixture was spun down and supernatant was removed and neutralized by addition of 30µl 1M Tris pH 8. The protein was precipitated using methanol/chloroform protein extraction. 4 volumes of methanol, 1 volume of chloroform and 3 volumes of water, all ice cold, were added and mixed thoroughly by vortexing, and incubated on ice for 15 minutes. The mixture was spun down in a tabletop centrifuge for 15 minutes at maximum speed. The top layer of resulting mixture was carefully removed, taking care not to disturb the interphase. 3 volumes of methanol is added and mixed thoroughly by vortexing, and spun down in a tabletop centrifuge for 5 minutes at top speed. The supernatant was removed leaving

the precipitated protein, which was allowed to dry and then resuspended in SDS-PAGE sample buffer. Following electrophoresis, gels were fixed in 10% methanol and 30% glacial acetic acid in distilled water for 1 hour, and incubated in ENH^3ANCE containing radioactive tritium (NEN life sciences) for 1 hour. Gels were washed and incubated in cold water for 30 minutes and dried in a vacuum gel dryer for 2~3 hours. Dried gels were exposed to Kodak XS-R films overnight at -70°C and developed the following day.

4. Purification of mitochondria from mouse brain and cultured cells

Mice were sacrificed on the day of the experiment and their brains were removed and washed twice in ice-cold PBS. Cultured cells were lifted from the plates using PBS-citrate with 50mM EDTA. They were washed twice in PBS and resuspended in 1ml of ice- cold HIM buffer (200mM mannitol, 70mM sucrose, 10mM Hepes pH 7.5, 1mM EGTA, with protease inhibitors leupeptin, aprotinin, peptistatin, and PMSF) per 150mm plate for homogenization. 2ml of HIM buffer was used for homogenization per each mouse brain. Homogenization was carried out in a Dounce/Teflon homogenizer with 20 strokes. The resulting homogenate was spun down at 500xg for 4 minutes at 4°C. The initial pellet was saved as nuclear membrane

and cell debris (NM), and supernatant was taken and further centrifuged at 7000xg for 15 minutes at 4°C. The resulting pellet was saved as heavy membrane (HM) fraction. The supernatant was removed and spun at 17,000xg for 30 minutes, generating a cytosolic fraction (supernatant) and light membrane fraction (pellet).

Collected samples were mixed with SDS sample buffer and heated at 90°C for 5 minutes prior to separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto nitrocellulose membranes. After the completion of transfer, membranes were air dried for 5~10 minutes and blocked for 1 hour in TBST (10mM Tris-HCl pH 8, 150mM NaCl, and 0.05 Tween 20) containing 5% skim milk powder. Primary antibody was incubated with the membranes overnight in 4°C on a shaker. Membranes were washed in TBST 3 times for 10 minutes each. Following the wash, the membranes were incubated in secondary antibody for 1~3 hours, and washed in TBST in the same manner as before. Enhanced Chemiluminescence (NEN Perkin Elmer Life sciences Inc.) was used to detect the protein of interest according to the manufacturer's protocol. Primary antibodies were diluted as follows: α-CNP mouse monoclonal antibody 1:10,000~1:30,000, α-cytochrome Oxidase subunit IV mouse monoclonal antibody 1:2500, both diluted in 1% skim milk powder+TBST. Horse radish peroxidase

conjugated goat α-mouse antibody was used as the secondary antibody for all cases diluted in 1:10,000, in and in 1% skim milk powder+TBST.

4. In-Vitro binding and import assay of CNP2

Various CNP isoforms and their permutations were translated in-vitro from CNP cDNAs in pBluescript SK (T3 promotor) using the T_NT coupled rabbit reticulocyte lysate system (Promega) with S35-labeled methionine and cysteine, according to the manufacturer's protocol. 5µl of the translation mixture was incubated with 25µl of HM fraction from mouse brain resuspended in cMRM buffer (250mM sucrose, 10mM Hepes pH 7.5, 1mM ATP (K^+ salt), 5mM sodium succinate, 0.08mM ADP, 2mM K_2PO_4), along with 20µl of Buffer A (20mM Hepes-KOH, 10mM KCl, 2.5mM $MgCl_2$) for half an hour at 30°C. The resulting mixture was layered onto a cushion of 500µl MRM (250mM sucrose, 10mM Hepes-KOH pH 7.5) and centrifuged at top speed in a microcentrifuge for 4 minutes. The majority of supernatant was aspirated, followed by centrifugation for 2 additional minutes. The resulting supernatant was carefully removed with a pipette. The pellet was resuspended in 1X SDS sample buffer and heated at 95°C for 5 minutes prior to SDS-PAGE analysis.

5. Protease treatment of purified mitochondrial pellet

Heavy membrane fraction from HeLa S3 cells transfected with M21L was obtained as mentioned above. The pellet was suspended in cMRM to a concentration of 0.5μg/μl according to Bradford assay. 100μl of the heavy membrane mixture was used per reaction. For samples containing permeablized mitochondria, final concentration of 1% Triton X-100 was added to the reaction mixture prior to protease treatment. 25μl of trypsin or 5μl of Proteinase K were put into each tube, followed by incubation for 30 minutes at 37°C. The reaction was stopped by adding 25μl of soybean trypsin inhibitor for trypsin or 5μl of PMSF for Proteinase K and mixed thoroughly. SDS sample buffer was added to each sample and boiled for 5 minutes prior to analysis by SDS-PAGE.

3.3 Results

Immunofluorescence analysis of various CNP constructs

Expression of CNP2 in HeLa S3 resulted in mitochondrial localization of CNP. Confocal images of HeLa S3 transfected with M21L construct show clear overlap of CNP staining and MitoTracker, a widely used mitochondrial marker (figure 3-1). As expected, mutations of serine 9 and 22 to alanine show strong localization of CNP to mitochondria, whereas mutation to aspartic acid abolishes the localization (figure 3-1). Also, expression of GFP tagged with the N-terminal 20 amino acids of CNP2 show strong mitochondrial localization (figure 3-2).

Transfection of HeLa S3 cells with M21L to produce CNP2, resulted in approximately 40% of the population showing CNP2 exclusively localized to mitochondria, and 15% of the population showing no signs of mitochondrial localization (figure 3-3, control). The rest of the transfected cells showed CNP2 diffused in cytoplasm as well as localized to mitochondria. The efficiency of FuGENE varied from one trial to another, causing fluctuation of protein expression level. Cells with high expression of CNP2 display, in addition to mitochondrial localization of CNP2, a typical phenotype of CNP1 expression, namely morphological changes with

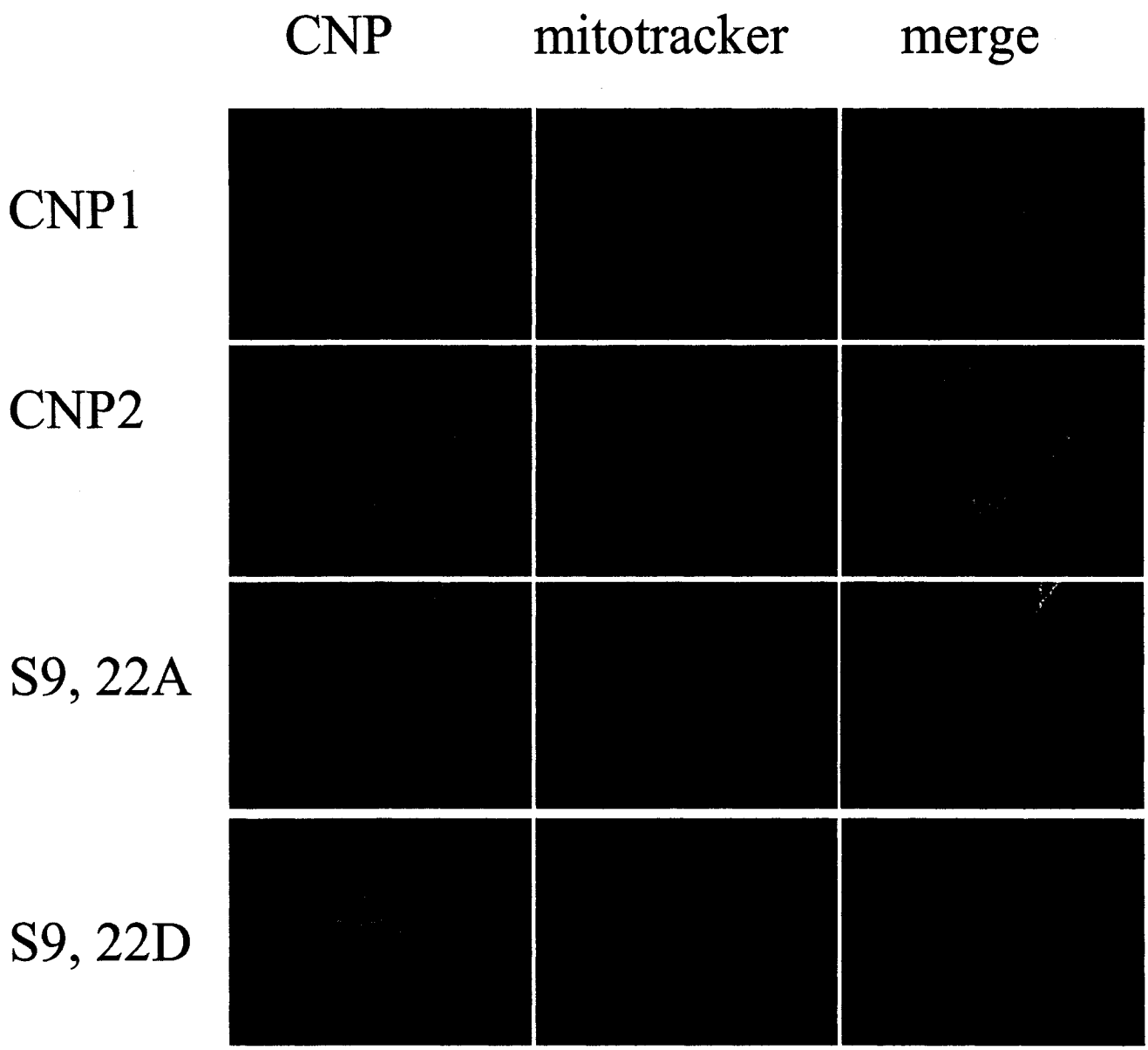

CNP
mitotracker
merge
CNP1
CNP2
S9, 22A
S9, 22D

Figure 3-1: Various CNP constructs expressed in HeLa cells

HeLa S3 cells were transiently transfected using FuGENE transfection reagent. Cells were stained using α-CNP mouse antibody and Alexa 488-conjucated goat anti-mouse secondary antibody (green), and MitoTracker Red CMXRos (Red). Expression of CNP1 results in visualization of the cell membrane. CNP2 clearly displays localization to mitochondria, as seen in the overlap of the two fluorofores, resulting in yellow color in the merged image. S9, 22A shows patterns identical to CNP2, whereas S9, 22D closely resembles CNP1.

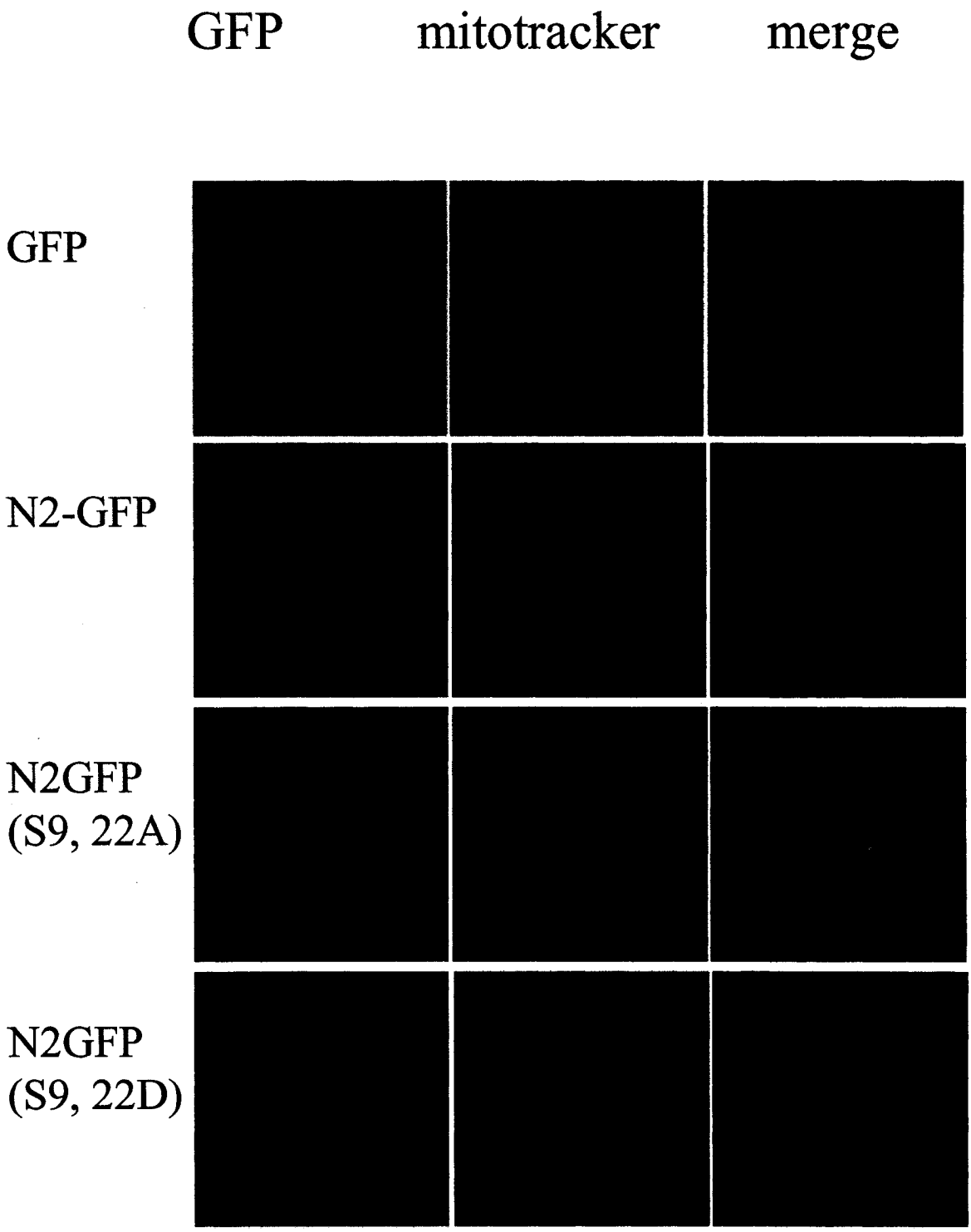
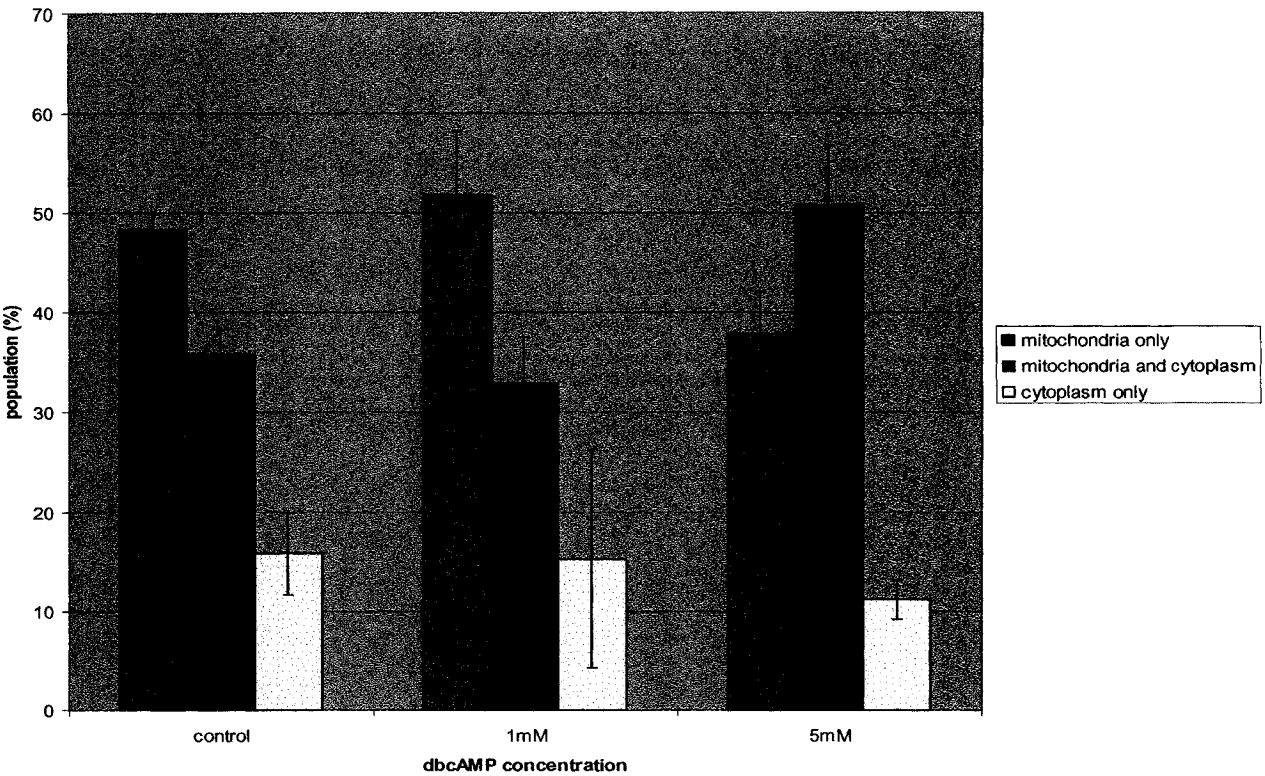

GFP
mitotracker
merge
GFP
N2-GFP
N2GFP
(S9, 22A)
N2GFP
(S9, 22D)

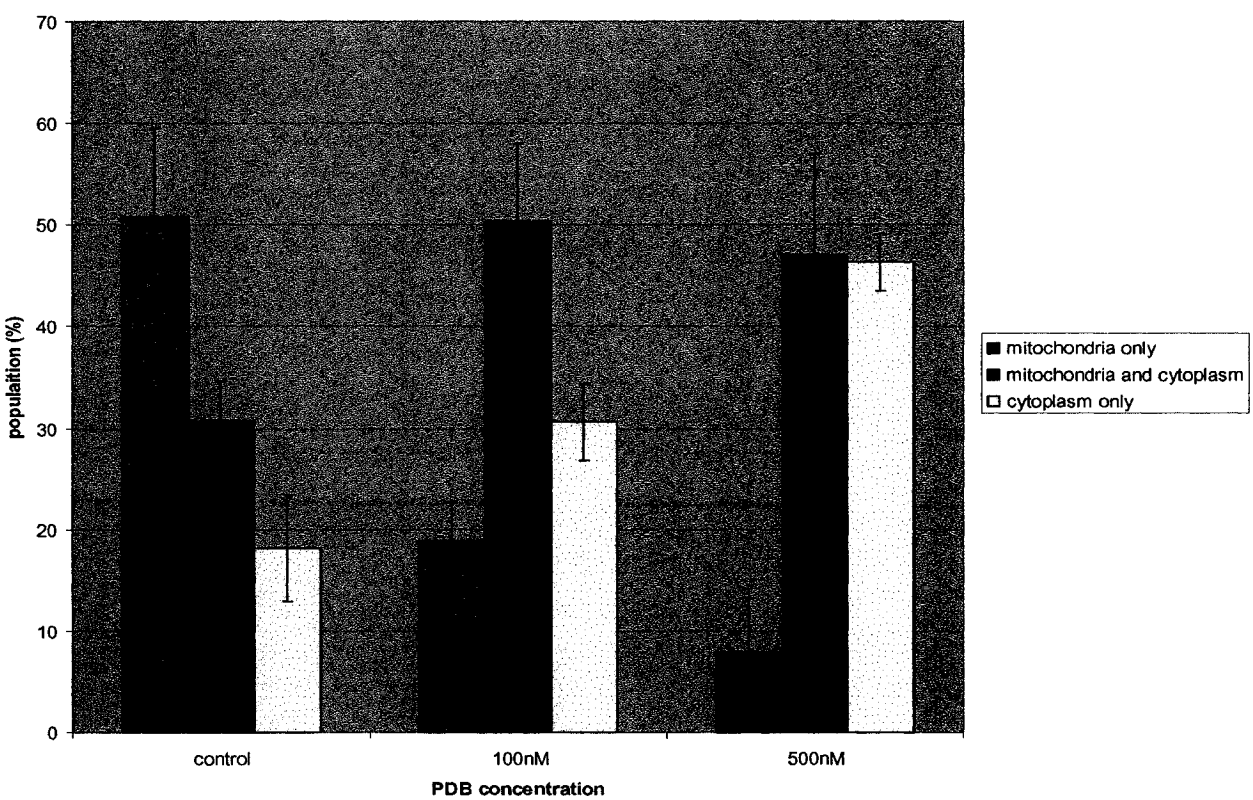
Figure 3-2: N2-GFP expression in HeLa S3 results in mitochondrial localization.

HeLa S3 were transiently transfected with N2-GFP using FuGENE transfection reagent. Cells are stained using MitoTracker Red CMXRos (red). Expression of GFP alone results in illumination of the entire cell. However, when GFP is tagged with the N2, it is mostly localized to mitochondria. Serine mutants of N2-GFP show comparable results to those of M21L serine mutants.

A


70
60
50
40
30
20
10
0
population (%)
control
1mM
5mM
dbcAMP concentration
mitochondria only
mitochondria and cytoplasm
cytoplasm only

B


70
60
50
40
30
20
10
0
population (%)
control
100nM
500nM
PDB concentration
mitochondria only
mitochondria and cytoplasm
cytoplasm only

C

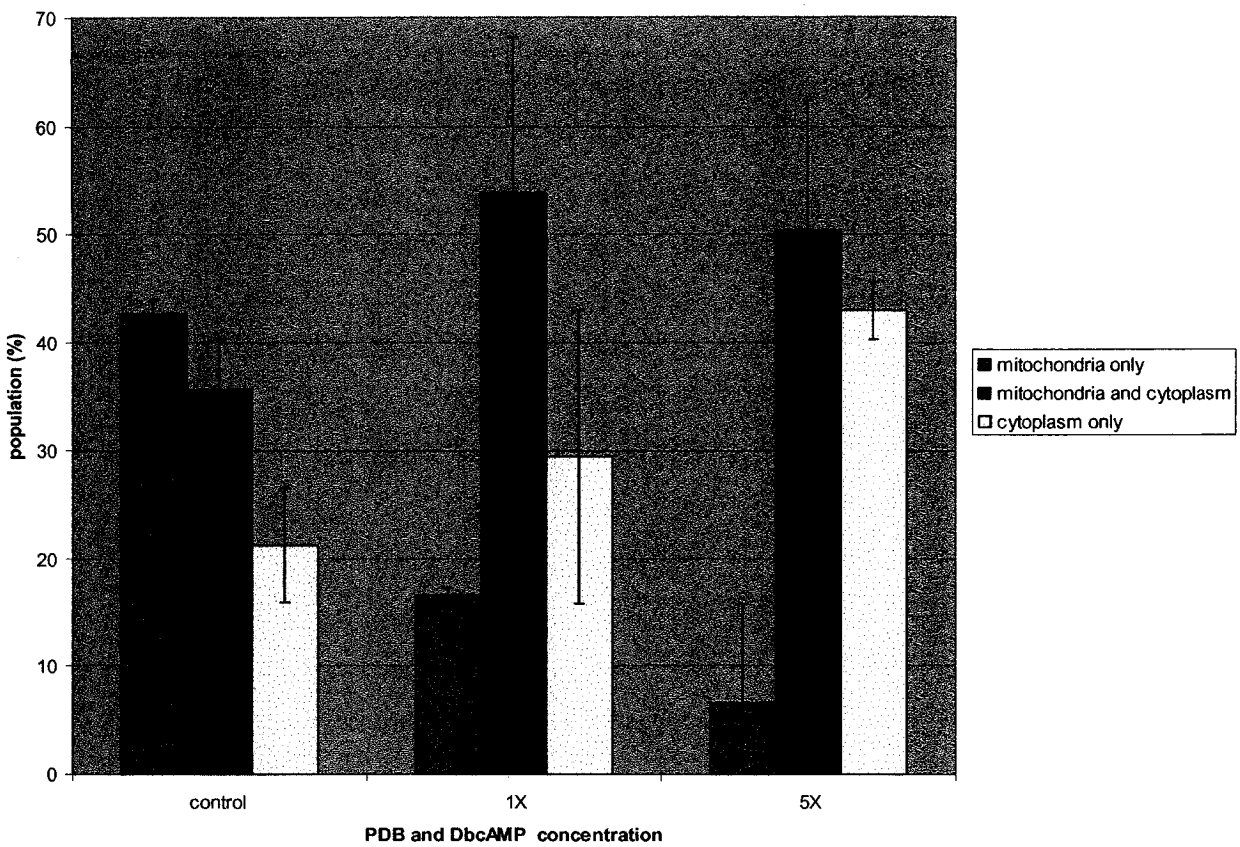

70
60
50
40
30
20
10
0
population (%)
control
1X
5X
PDB and DbcAMP concentration
mitochondria only
mitochondria and cytoplasm
cytoplasm only

Figure 3-3: distribution of HeLa S3 cells transfected with RCCMV CNP2 M21L and treated with kinase activators for 18 hours.

HeLa S3 were transiently transfected with M21L using FuGENE transfection reagent, and treated with kinase activators for 18 hours. Cells were fixed and stained with anti-CNP antibody and anti COX-IV antibody, and the numbers are counted into three categories using fluorescence microscopy: showing CNP strictly localized to mitochondria (mitochondria only), CNP both on mitochondria and in cytoplasm (mitochondria and cytoplasm) and CNP showing no signs of mitochondrial localization (cytoplasm only). Typical distribution of M21L transfected cells show approximately 40~50% of total transfected cells displaying CNP2 localized strictly to mitochondria, and less than 20% without any signs of mitochondrial localization. The remaining population of cells, approximately 30%, displayed both characteristics. Treatment of cells with DbcAMP results in little changes in the distribution of cell population in the above three categories (A). Treatment of cells with PDB results in delocalization of CNP from mitochondria (B). At 500nM, PDB increases the population of cells without mitochondrial localization of CNP from approximately 18% to 45%. Simultaneous administration of PDB and DbcAMP (C) results in loss of mitochondrial localization similar to that of PBD administration alone (B).

extensive filipodia formation. This is likely due to exceeding the threshold of CNP2 which can be localized to mitochondria, causing CNP to accumulate in the cytoplasm and behave like CNP1. The ratio of cells showing strictly mitochondrially localized CNP and those that also showed cytoplasmic distribution of CNP inevitably varies with the fluctuations in the expression level and transfection efficiency. However, in all experiments, the population of cells that do not display any signs of mitochondrial localization of CNP remains relatively constant. On the contrary, endogenous CNP in both HeLa S3 (unpublished data) and adrenal medullary chromaffin cells (McFerran et. al., 1997) primarily localized to mitochondria. Detection of endogenous CNP in HeLa S3 was achieved using biotin/stereptavidin conjugated fluorofore which amplified the signal (data not presented due to fast fading of fluorofore). The endogenous expression of CNP in these cells is likely to be controlled tightly, resulting in efficient mitochondrial localization without excessive build-up of CNP in the cytoplasm.

As the consensus sequences of PKA and PKC phosphorylation sites resemble that of amino acid sequences surrounding the two serine residue in the N-terminal extension of CNP2 subtype, activators of the two kinases were used to treat transfected cells overnight (figure 3-3). N, 2'-O-Dibutyryladenosine-3'-5-cyclic monophosphate (DbcAMP) treatment of HeLa S3 transfected with M21L did not result in any noticeable

trend. The population of cells showing clear mitochondrial localization differed from drug treated to non-treated cells only by small and random fluctuations, both at 1mM and 5mM. However, with the use of phorbol 12, 13-dibutyrate (PDB), there were noticeable changes. At the concentration of 100nM, the population of cells whose CNP staining indicates exclusively mitochondrial localization, decreased from approximately 50% to 20%. At 500nM of PDB, this population diminished to 10% or less. Without PDB treatment, the proportion of cells showing no visible mitochondrial localization was less than 20%. After PDB treatment, this increased to approximately 30% and 45% at 100nM and 500nM respectively. Double treatment using dbcAMP and PDB was attempted as well, which resulted in effects similar to the treatment with PDB alone. This initially suggested that PKC was the primary kinase involved in the regulation of CNP2 in its localization to mitochondria. Subsequently, analogues of dbcAMP and PDB were tested in order to further confirm the kinase involved in this phosphorylation event. Sp-cAMPs, which is an analogue of cAMP, hence acting as a PKA activator, resulted in no apparent changes to the localization of CNP2 to mitochondria, either at 1mM or 5mM (results not shown). However, two phorbol esters, PDD and PMA which activate PKC, showed effects which were similar to that of PDB (figure 3-4). The population of cells showing mitochondrially localized CNP2

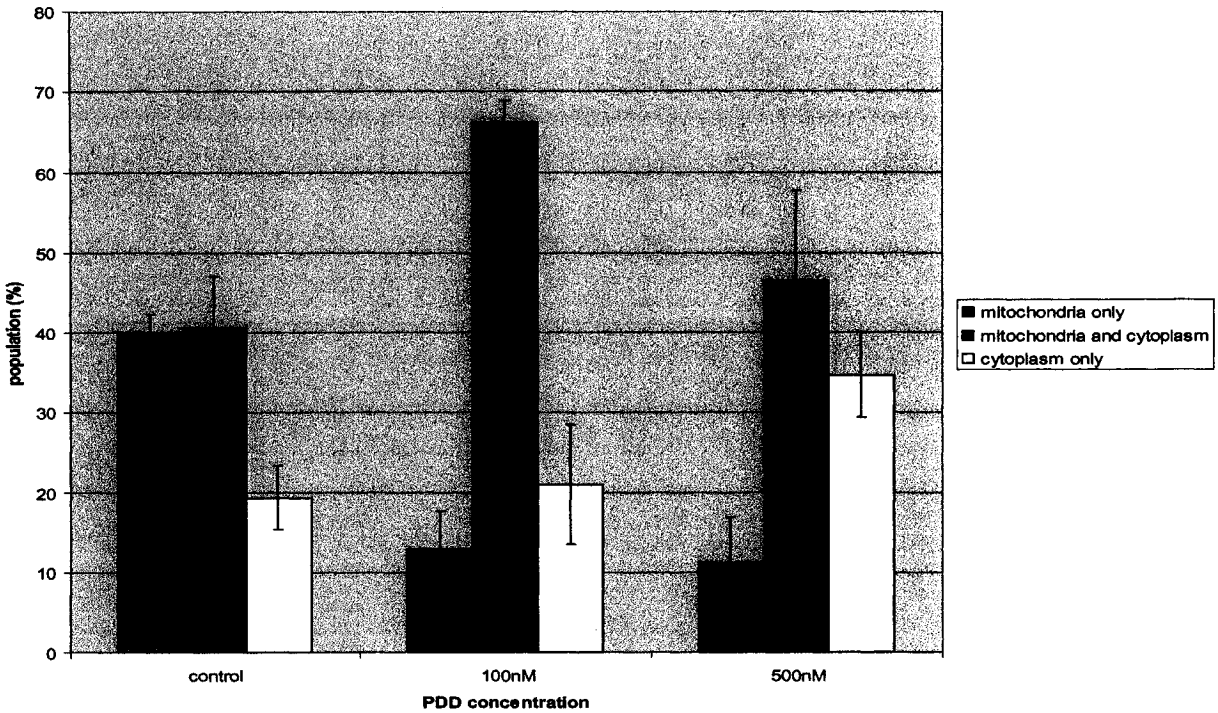
decreases from 40% to less than 10% following PDD treatment at 500nM. Also, cells showing exclusively cytoplasmic CNP increase from 20% of control to 40% in 500nM PDD. A similar pattern is observed in PMA treated cells, indicating that activation of PKC results in localization of CNP2 away from mitochondria, and phosphorylation by the kinase could be the key event in the regulation of mitochondrial localization.

Although cells were incubated with kinase activators overnight, short-term effects of these drugs were also investigated (figure 3-5). Surprisingly, both DbcAMP and PDB had similar effect on the localization of CNP to mitochondria. Both drugs showed maximum effect after 1-hour incubation. DbcAMP caused an increase in the population of cells showing only cytoplasmic CNP, from 15% to more than 35%, after 1 hour of treatement. PDB exerted similar effects, peaking at the treatment time of 1 hour. However, PDB was more persistent than DbcAMP, as the effect did not fully diminish even after an overnight treatment.

Analysis of CNP2 mutants: investigation of amino acid residues targeted for post-translational modification

Serine mutants of RcCMV CNP2 M21L were tested in order to examine which serine residue, if not both, was being phosphorylated by PKC, leading to

A


80
70
60
50
40
30
20
10
0
population (%)
control
100nM
500nM
PDD concentration
mitochondria only
mitochondria and cytoplasm
cytoplasm only

B

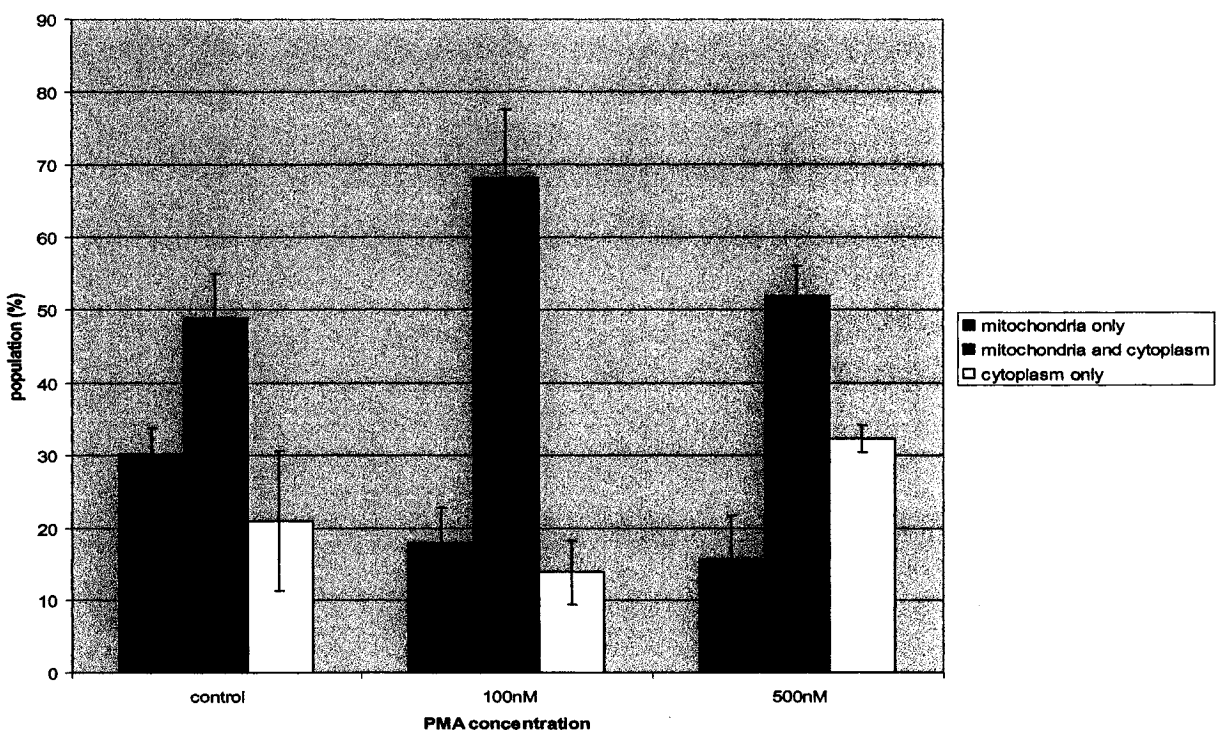
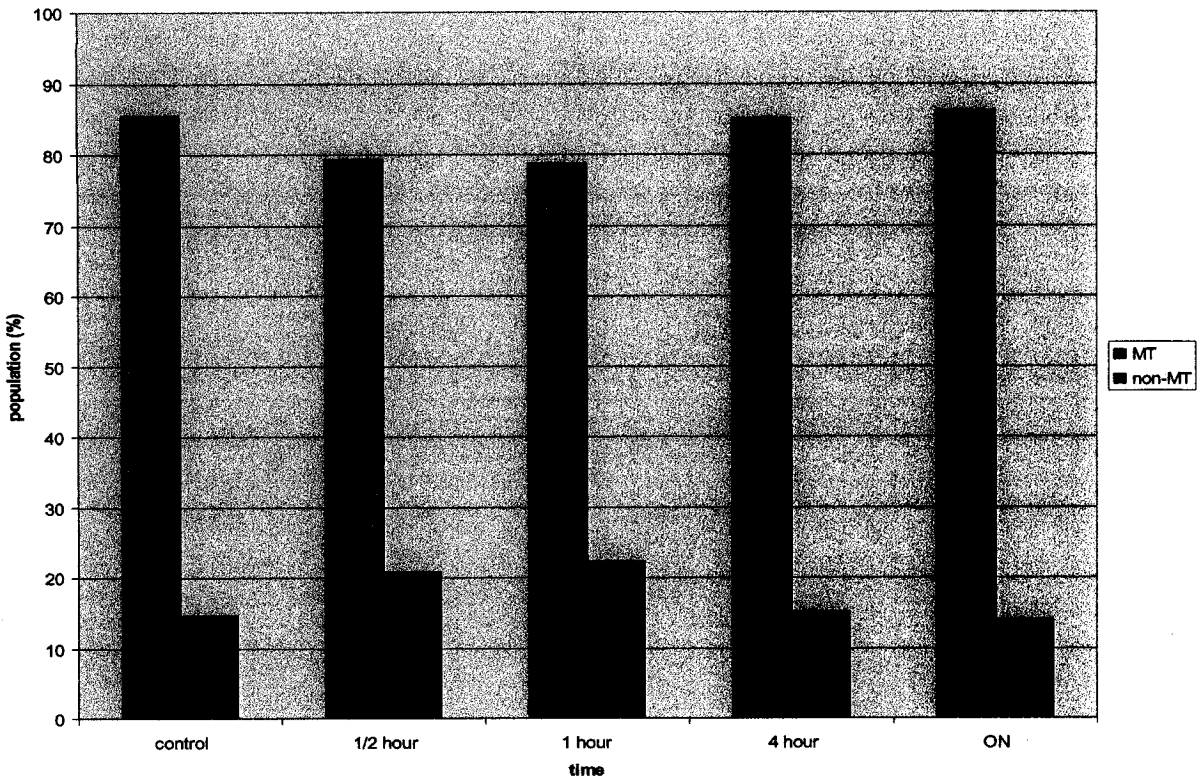

90
80
70
60
50
40
30
20
10
0
population (%)
control
100nM
500nM
PMA concentration
mitochondria only
mitochondria and cytoplasm
cytoplasm only

Figure 3-4: Treatment of HeLa S3 transfected with M21L with analogues of PDB. Transfected HeLa S3 cells were subjected to treatments with PDD and PMA for 18 hours, and changes were observed by statistical analysis. Both PDD and PMA yielded results similar to that of PBD. Treatment of cells with PDD resulted in decrease of mitochondrially localized CNP2 from 40% (control) to 12% (500nM). Likely, treatment of PMA resulted in decrease from 31% to 17%.

A


100
90
80
70
60
50
40
30
20
10
0
population (%)
control
1/2 hour
1 hour
4 hour
ON
time
MT
non-MT

B

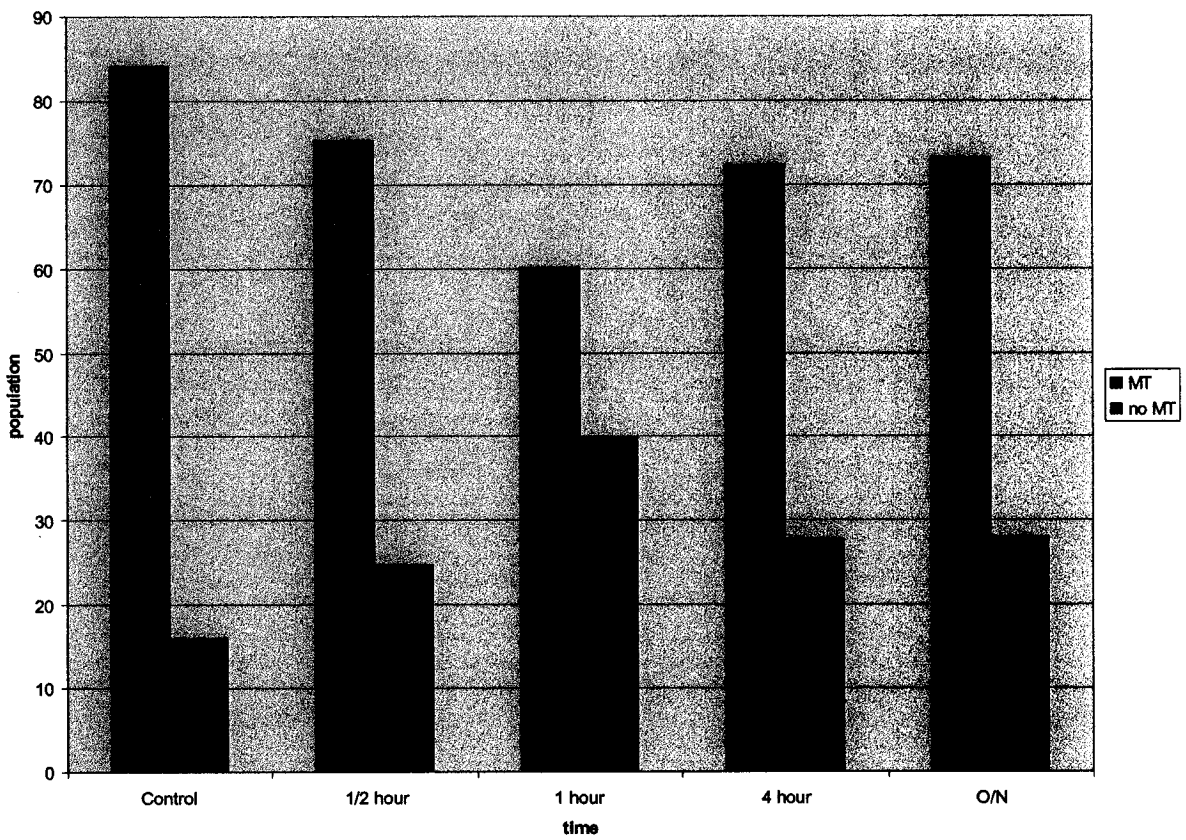

90
80
70
60
50
40
30
20
10
0
population
Control
1/2 hour
1 hour
4 hour
O/N
time
MT
no MT

Figure 3-5: Treatment of HeLa S3 transfected with M21L with dbcAMP (A), and PDB (B) for various lengths of time.

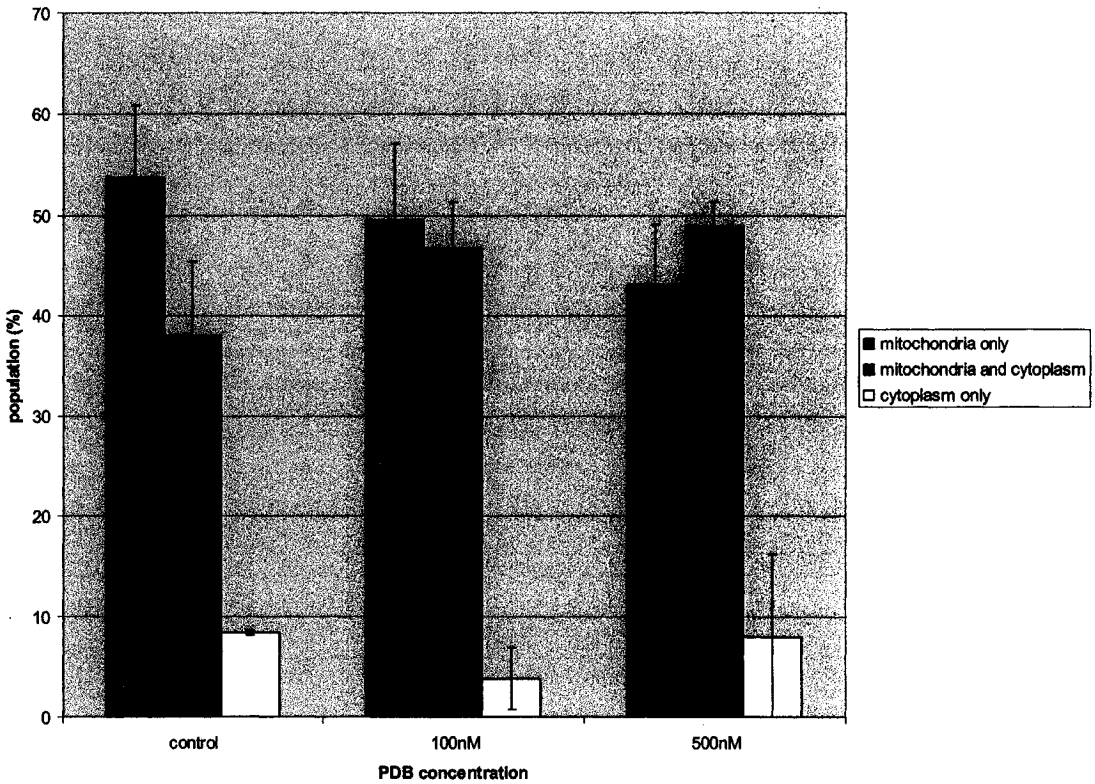
Transfected HeLa S3 cells were subjected to treatments with dbcAMP and PDB for various time lengths, and the result from a single experiment is shown. For simplicity, cells were counted into two groups, those that show mitochondrial localization of CNP (equivalent to mitochondria only and mitochondria and cytoplasm in previous figures), and those that do not show any signs of mitochondrial localization of CNP (equivalent to cytoplasm only in previous figures). Both DbcAMP and PDB exerted comparable effects, peaking at 1 hour of treatment. However, the effects of PDB persisted at approximately 70% of the maximal effect overnight, whereas the effect of DbcAMP disappears after overnight incubation.

non-mitochondrial localization of CNP. Two different serine residues at the N-terminus of M21L sequence were replaced by to alanine, individually (S9A, S22A) and simultaneously (S9, 22A). After 6 hour transfection, cells were treated with PDB under the same conditions as stated above. As expected, S9, 22A was not at all affected by the presence of PDB in either concentration (figure 3-6). Additionally, elimination of serines resulted in a higher population of cells showing exclusively mitochondrially localized CNP, suggesting increased efficiency of mitochondrial localization due to prevention of phosphorylation. Moreover, single serine replacement by alanine (S9A, S22A) did not show any apparent delocalization from mitochondria after treatments with PDB. This suggests that phosphorylation of a single serine residue does not result in inhibition of CNP2 localization to mitochondria, and both residues are required to be phosphorylated in order to maintain CNP2 in the cytoplasm. DbcAMP treatment of the mutants were not attempted because phosphorylation of N2 by PKA was not suspected at the time.

Mutation of cysteine 417 to serine affected mitochondrial localization as well. Isoprenylation of cysteine 417 has been known to facilitate membrane localization of CNP (Braun et. al, 1991). Elimination of cysteine resulted in approximately half of the transfected cells showing no mitochondrial localization of CNP, an increase of

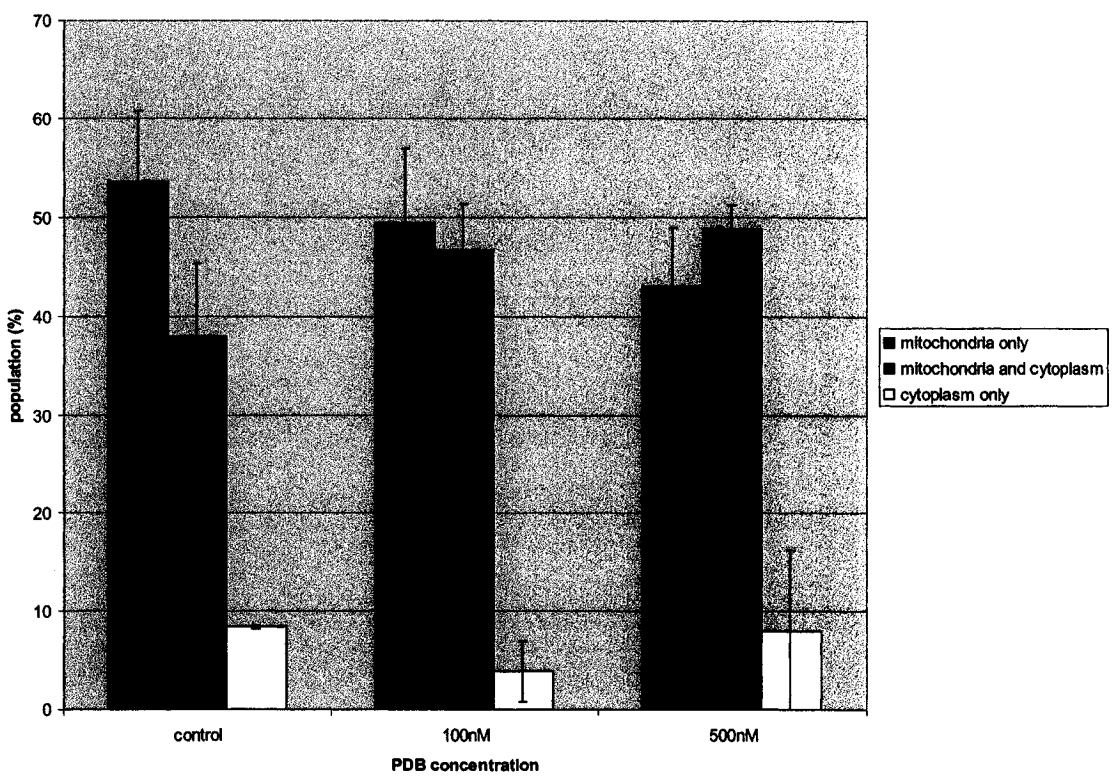
A

S9A


70
60
50
40
30
20
10
0
population (%)
control
100nM
500nM
PDB concentration
mitochondria only
mitochondria and cytoplasm
cytoplasm only

B

S22A


70
60
50
40
30
20
10
0
population (%)
control
100nM
500nM
PDB concentration
mitochondria only
mitochondria and cytoplasm
cytoplasm only

C

S9,22A

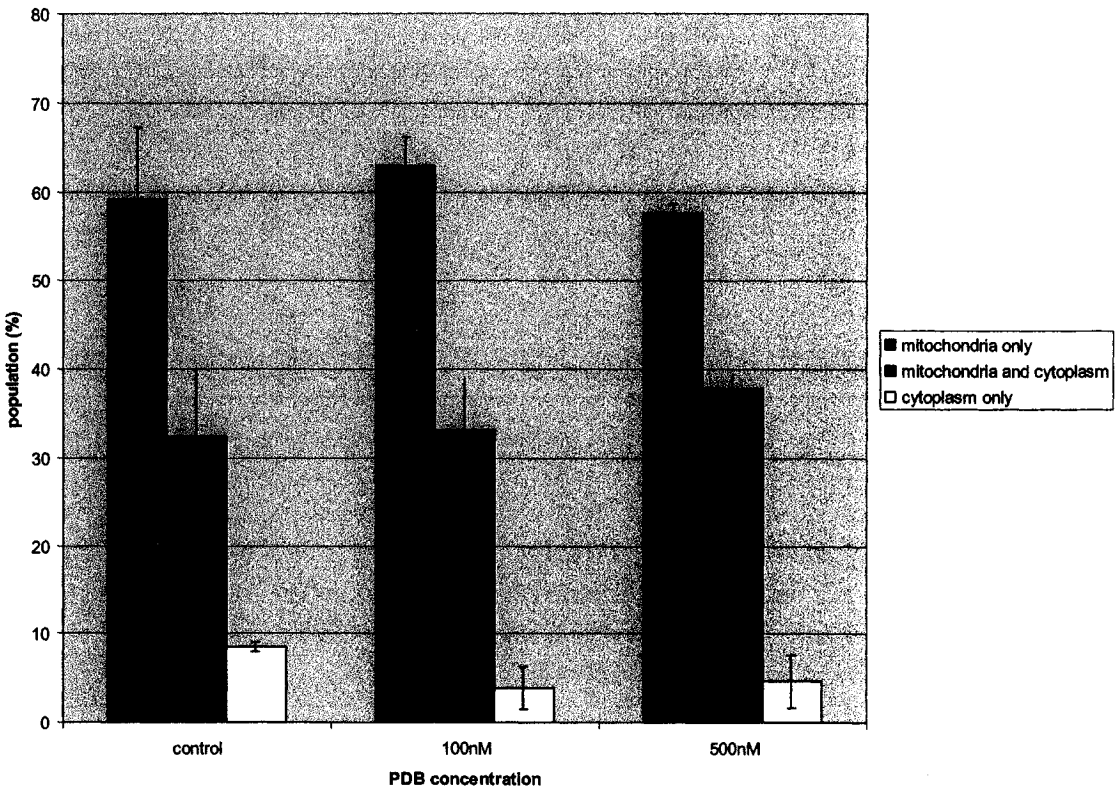

80
70
60
50
40
30
20
10
0
population (%)
control
100nM
500nM
PDB concentration
mitochondria only
mitochondria and cytoplasm
cytoplasm only

Figure 3-6: Treatment of HeLa S3 transfected with S9A (A), S22A (B) and S9, 22A (C) with PDB for 18 hours.

HeLa S3 cells Transfected with CNP2 mutants were subjected to treatments with PDB, and changes were observed by statistical analysis. The population of cells showing mitochondrial localization of CNP is higher than that of M21L transfected cells, even without treatment with PDB. PDB treatment of S9A, S22A and S9.22A transfected cells did not exert any effect on the localization of the expressed proteins.

approximately 30~35% (figure 3-7). Treatment with PDB resulted in further non-mitochondrial localization of CNP, although the change was not as dramatic as in M21L transfected HeLa S3.

Pulse Chase of M21L in COS-7 and HeLa S3

N-terminal mitochondrial matrix targeting sequences are usually cleaved by the mitochondrial matrix peptidases upon reaching the target location. Labeling proteins with radioactive isotopes and monitoring changes in molecular weight due to processing, is a conventional method of investigating whether mitochondrial import is taking place. As the cleavage of CNP2 is expected to take place after lysine 20, processing of CNP2 in this way would generate what is essentially CNP1. Experiments were done using two different cell lines, COS-7 which do not show mitochondrial localization of CNP, and HeLa S3 which shows good mitochondrial localization of CNP. As expected, CNP2 did not degrade in COS-7 cells (figure 3-8 A). Large quantities of protein were expressed by transfecting COS-7, resulting in clean immunoprecipitation of CNP. There was no sign of CNP2 degradation indicating that there is no processing of the N2. The identical experiment was repeated with HeLa S3 cells. CNP1 and CNP2 from COS-7 cells were used as a molecular weight references (lanes 8 and 9, figure 3-8 B).

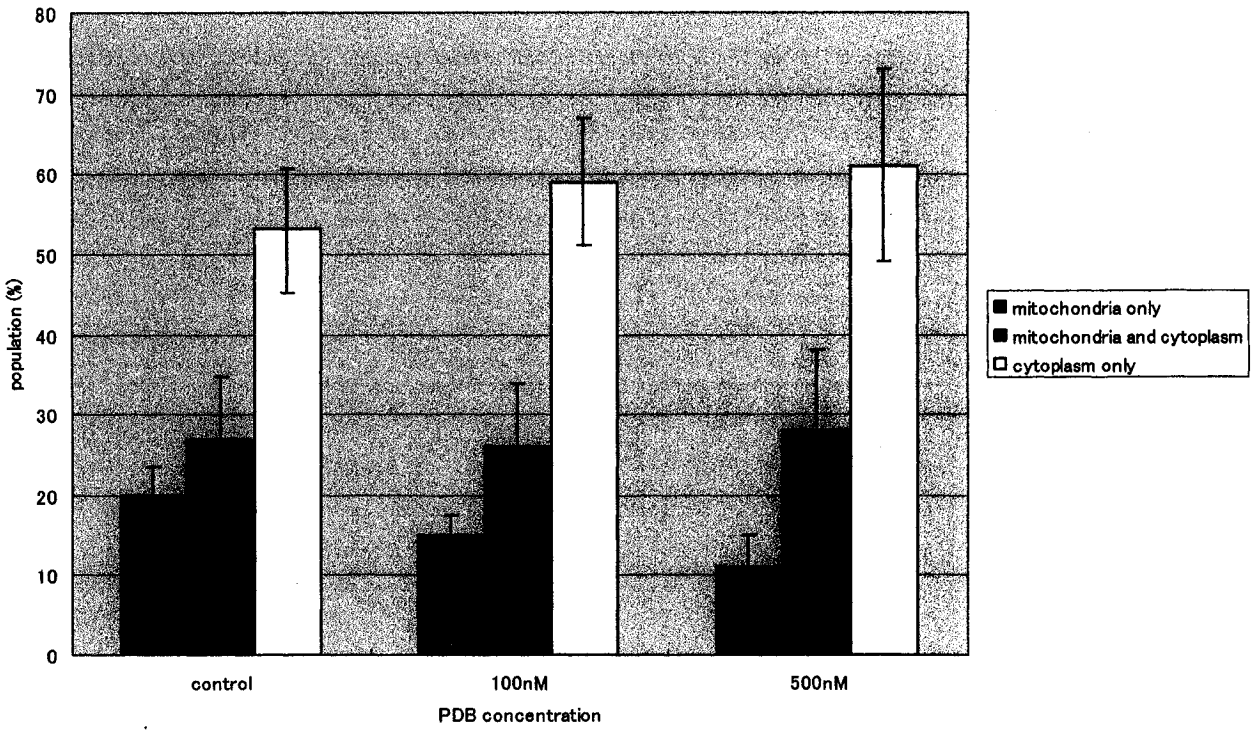

80
70
60
50
40
30
20
10
0
population (%)
control
100nM
500nM
PDB concentration
mitochondria only
mitochondria and cytoplasm
cytoplasm only

Figure 3.7: Treatment of HeLa S3 transfected with C417S with PDB for 18 hours.

HeLa S3 were transiently transfected with C417S using FuGENE transfection reagent, and treated with PDB for 18 hours. Cells were processed and counted using fluorescence microscopy. Change of isoprenylation target, cysteine 417 of CNP2, to serine results in decreased mitochondrial localization. PDB treatment of these cells further decreased mitochondrial localization of C417S.

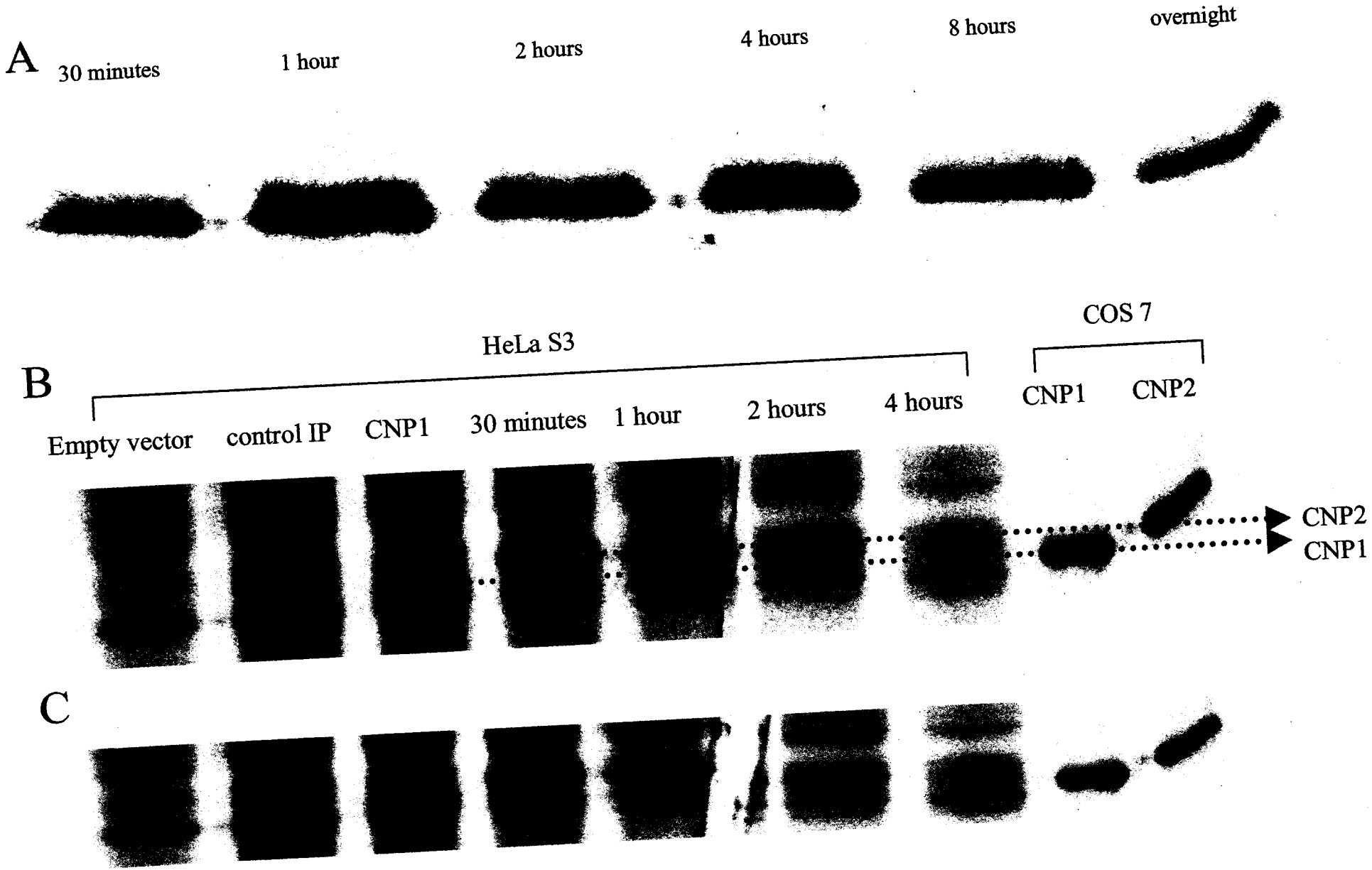

A
30 minutes
1 hour
2 hours
4 hours
8 hours
overnight
B
HeLa S3
COS 7
Empty vector
control IP
CNP1
30 minutes
1 hour
2 hours
4 hours
CNP1
CNP2
CNP2
CNP1
C

Figure 3.8: Pulse and chase of CNP2 in COS-7 cells (A) and HeLa S3 cells: edited (B), unedited (C).

A: Pulse-chase of CNP2 in Cos-7 cells. M21L transfected COS-7 cells were incubated in serum, methionine and cysteine free DMEM for half an hour and labeled with S35 methionine and cysteine for 5 minutes. Cells were washed in PBS and incubated in DMEM containing 10% fetal bovine serum and harvested at indicated time points. The lysates of harvested cells were immunoprecipitated using α-CNP mouse antibody and analyzed by SDS-PAGE. Obtained CNP samples show no signs of processing.

B: Pulse-chase of CNP2 in HeLa S3 cells. M21L transfected HeLa S3 cells were incubated in serum-, methionine- and cysteine-free DMEM for half an hour and labeled with S35 methionine and cysteine for 5 minutes. Cells were washed in PBS and incubated in DMEM containing 10% fetal bovine serum and harvested at indicated time points. The lysates of harvested cells were immunoprecipitated using α-CNP mouse antibody, and analyzed by SDS-PAGE. Immunoprecipitation was tested using normal mouse antibody to test the specificity of the process (lane control IP). CNP 1 and 2 expressed in COS-7 cells were run on the same gel as molecular weight references (two lanes on the right side). Obtained CNP samples show no signs of processing.

C: unedited photo of pulse-chase of CNP2 in HeLa S3 cells.

Due to the low transfection efficiency, CNP2 is detected in low quantity with many contaminating bands in the background. Despite the prediction of N2 as a mitochondrial matrix targeting signal, no processing was observed in HeLa S3 cells. CNP2 did not change in its molecular weight, and the signal eventually faded below a detectable level.

Fractionation of mitochondria and in-vitro binding of CNP2

Utilizing the difference in density of various subcellular organelles, centrifugation is used as a method for isolation of mitochondria. Each fraction collected from the fractionation process was tested by α-CNP antibody and α-COXIV antibody. CNP was mostly detected in the nuclear membrane fraction (NM) containing the nucleus, unbroken cell debris and cell membrane, and in the heavy membrane fraction (HM) that includes mitochondria. Due to the localization of CNP to the plasma membrane, CNP was expected to be detected in the NM fraction (figure 3-9). However, there is an abundance of CNP in the HM fraction; hence it is clear that a significant amount of CNP is associated with mitochondria. Presence of COXIV in the NM fraction also indicates that mitochondria are present in unbroken cell debris. Most of the COXIV was detected in the HM fraction, as expected.

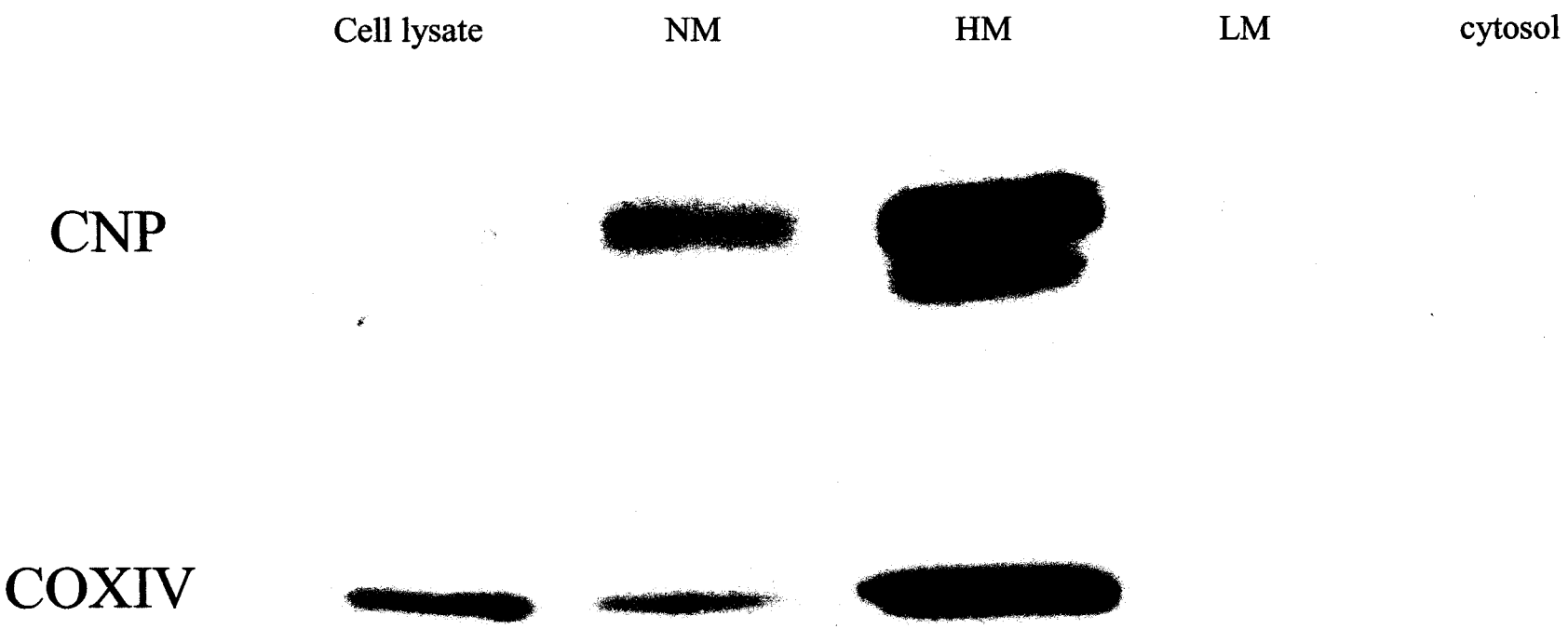

Cell lysate
NM
HM
LM
cytosol
CNP
COXIV

Figure 3.9: fractionation of HeLa S3 transfected with M21L.

HeLa S3 cells were transfected with M21L using FuGENE transfection reagent and incubated overnight. Cells were subsequently homogenized and fractionated using centrifugation. Samples of each fraction were collected, and are shown in the order of: whole cell lysate immediately after homogenation (cell lysate), nuclear membrane and unbroken cell debris (NM), heavy membrane fraction (HM), light membrane fraction (LM), and cytosol. Most of CNP and COX IV are found in the heavy membrane fraction, which includes mitochondria.

In vitro binding of CNP to mitochondria reveals that CNP2 associates with mitochondria. Without any mitochondria in the reaction mixture, both CNP1 and 2 were found primarily in the supernatant. With addition of mitochondria, the two proteins showed different patterns. CNP2 is found mostly in the mitochondrial pellet and little remains in the supernatant, whereas CNP1 is equally distributed in the mitochondrial pellet and supernatant (figure 3-10). An additional band of slightly lower molecular weight appeared after incubation with mitochondria. However, this band appeared both in CNP1 and CNP2 reaction mixture, indicating that this is not due to the processing of the N2 as a mitochondrial import signal.

Protease treatment of purified mitochondria

A trypsin treatment experiment was carried out according to the protocol of O'Neill (doctoral thesis, 2001), and the result was successfully replicated. Treatment of purified heavy membrane with trypsin did not degrade CNP unless the mitochondrial membranes were permeablized by addition of Triton X-100 (figure 3-11A). Identical samples probed with α-COXIV antibody, a protein which is located on the inner membrane of mitochondria, displayed the same pattern. This result led to the conclusion that CNP2 is imported into the mitochondrial matrix, thus is protected from

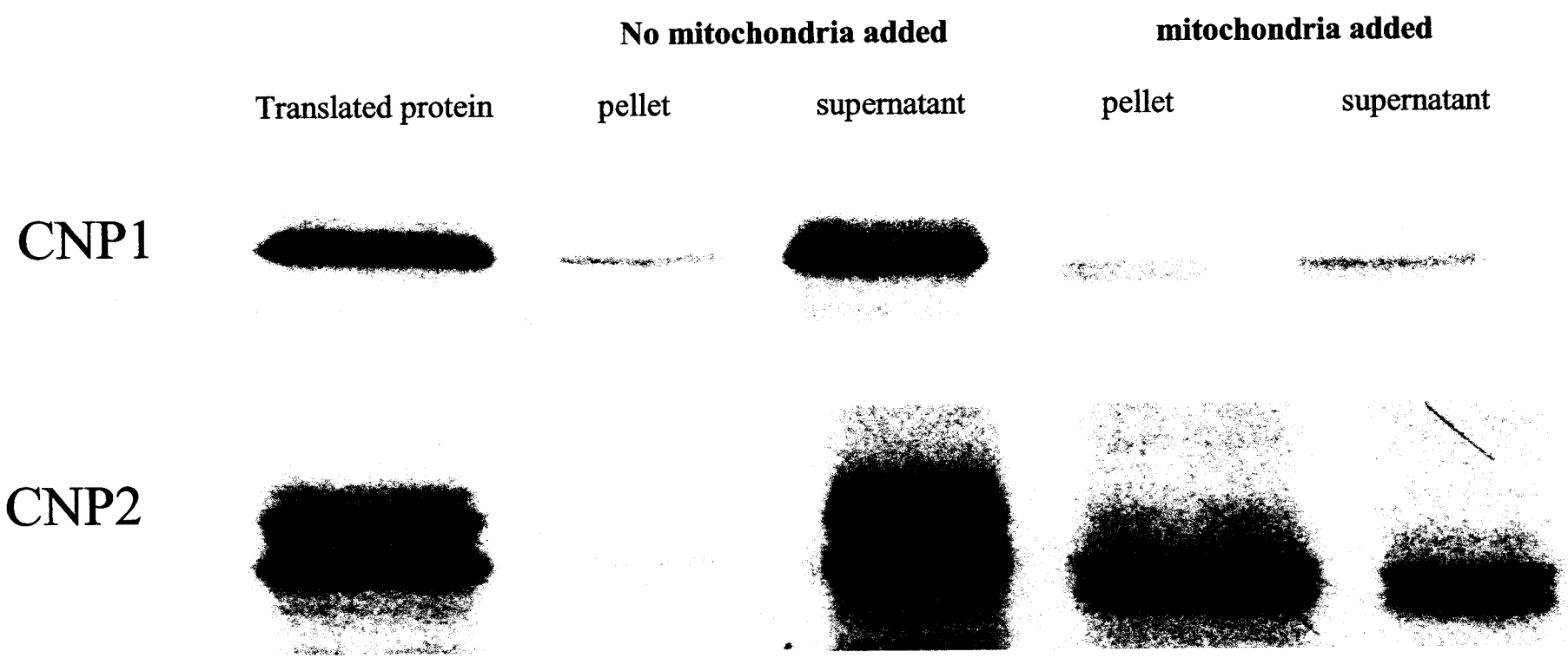
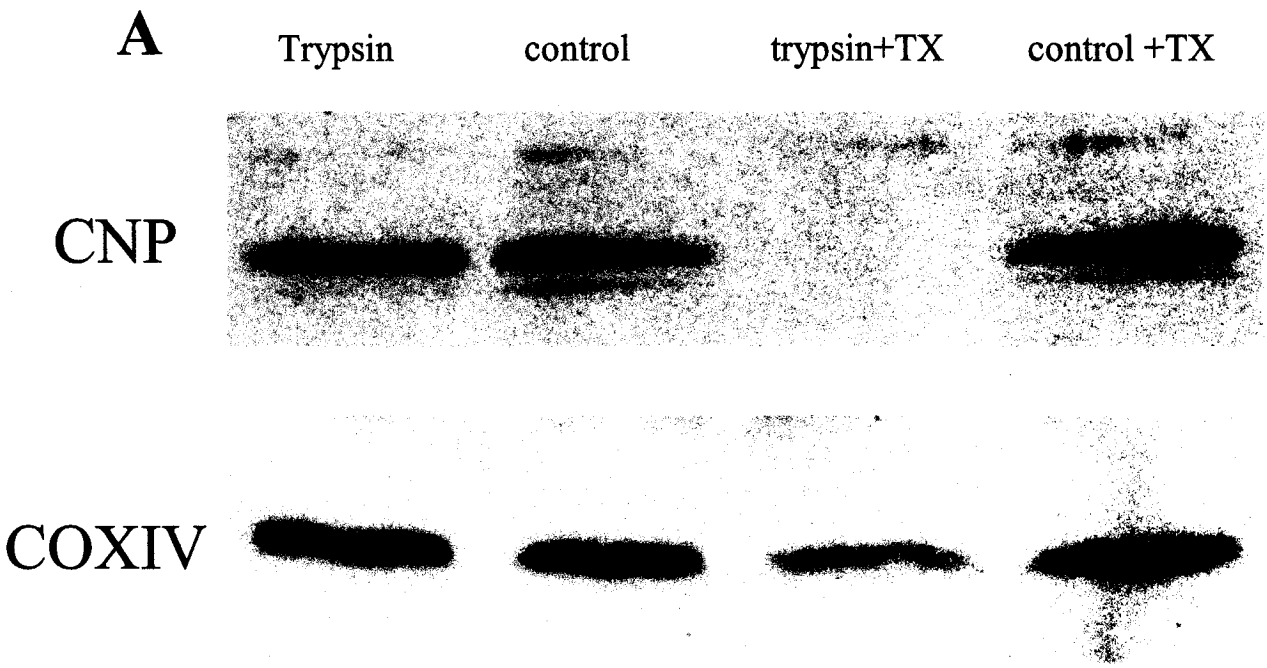

No mitochondria added
mitochondria added
Translated protein
pellet
supernatant
pellet
supernatant
CNP1
CNP2

Figure 3.10: In vitro- binding assay of CNP1 and CNP2 to mitochondria.

Heavy membrane fraction containing mitochondria was incubated with radioactively labeled CNP1 and CNP2 for 30 minutes at 30°C. The samples were run on a SDS-PAGE gel, and exposed to a film overnight at -70°C. The CNP2 translation mixture shows two bands prior to mixing with heavy membrane fraction, indicating that the lower band is not a species of CNP processed by mitochondria. Control reactions were carried out with the radioactive protein in the reaction mixture in the absence of mitochondria. CNP1 shows little affinity to mitochondria. CNP2, on the other hand, shows a significantly higher affinity to mitochondria, as shown in the pellet of the reaction containing mitochondria (lane 4). No signs of CNP2 specific processing is observed, as CNP1 and CNP2 samples show similar patterns when incubated with the heavy membrane fraction.


A
Trypsin
control
trypsin+TX
control +TX
CNP
COXIV

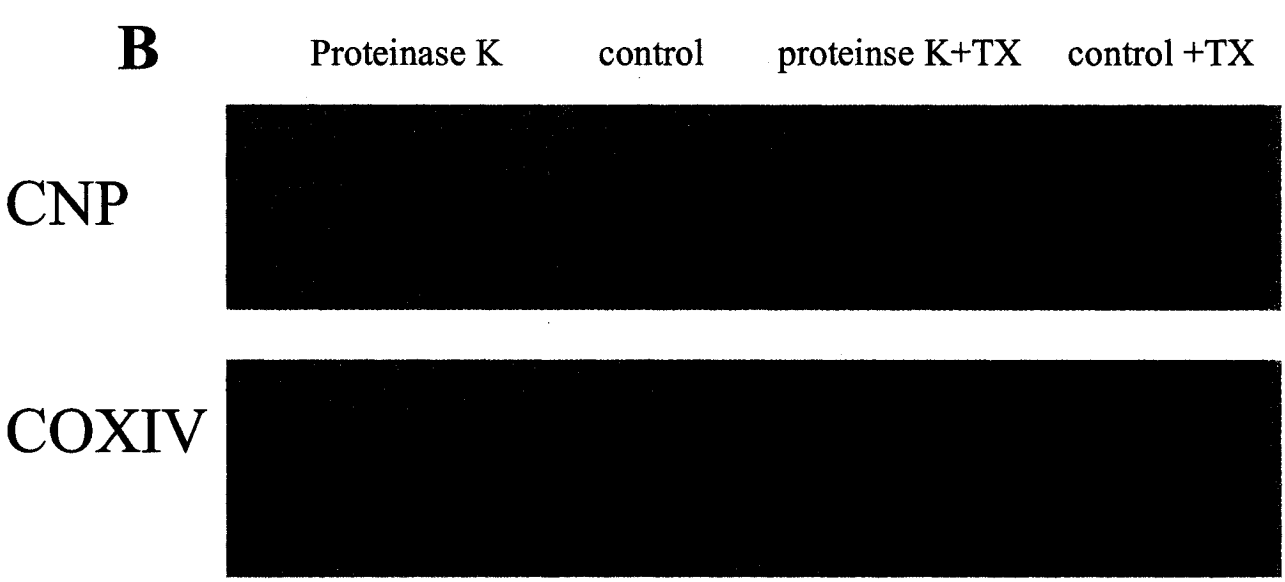

B
Proteinase K
control
proteinse K+TX
control +TX
CNP
COXIV

Figure 3-11: protease treatment of purified heavy membrane fraction of HeLa S3 transfected with M21L

Heavy membrane fraction from M21L transfected HeLa cells were treated with trypsin (A) or Proteinase K (B) in the absence or presence of a non-denaturing detergent (Triton X-100) which permeablizes the mitochondrial membrane. The samples are run on SDS-PAGE and analyzed using anti-CNP mouse antibody, and anti-COXIV mouse antibody. In both western blots, COXIV was used to test the integrity of mitochondrial membrane. It clearly displays that COXIV was protected from proteolysis when detergent was not present (lanes trypsin and Proteinase K), and is effectively degraded when detergent was used (lanes trypsin+TX and Proteinase K+TX).

A: CNP show resistance to trypsin (lane trypsin), and is degraded with the presence of detergent (lane trypsin+TX).

B: CNP is partially degraded in the presence of Proteinase K, even without detergent (lane Proteinase K), and completely degraded when detergent is used (lane Proteinase K+TX).

the protease. However, when the experiment was repeated with substitution of trypsin by Proteinase K, CNP is degraded partially or completely (figure 3-11 B). COXIV remained intact when the mitochondrial membrane was not permeablized with 1% Triton-X 100. Vulnerability of CNP2 to Proteinase K suggests CNP is exposed to the cytoplasm, and is not imported into the matrix.

3.4 Discussion

Much of the work I have presented are attempts to determine whether CNP2 isoform is imported into mitochondria or localized to the outer membrane by other means. The data collectively suggests that CNP is likely to be associated with mitochondria by means other than import. First, pulse-chase of CNP2 M21L shows no sign of processing. Two different methods were employed for expression of CNP2 in HeLa S3. Transient transfection was problematic due to low transfection efficiency. Expression of CNP2 was not abundant enough to allow clean immunoprecipitation, resulting in many contaminating bands in the final eluate. To address this issue, a viral vector was constructed by J. Lee. The proportion of cells expressing CNP2 is much greater with virally infected HeLa S3, and the resulting immunoprecipitation contained fewer contaminants. Results obtained from this experiment also show that there is no processing. Although there have been reports of mitochondrial protein whose import signals are not processed, they are usually embedded internally in the primary sequence, away from the termini. CNP does not possess such internal import signals, as indicated by failure of CNP1 to localize to mitochondria.

Secondly, protease treatment of purified mitochondria from CNP2-expressing cells show degradation of CNP. It was initially believed that CNP2 was imported due

to its resistance to degradation by trypsin. However, under identical experimental conditions, substitution of trypsin with Proteinase K, a more robust and undiscriminating protease, results in degradation of CNP. The apparent resistance to trypsin is likely due to a complex and dense tertiary structure or association with other proteins or membrane components that make the target sequence of trypsin protected from degradation. With either trypsin or Proteinase K, COXIV remained intact, showing the integrity of the mitochondrial membrane and the protection provided to the proteins on the inside. CNP showed slight resistance to Proteinase K as well, since complete disappearance of CNP band did not always occur. This is likely due to the same reasons for its resistance to trypsin, which is alleviated by presence of detergent that may act to disrupt tertiary structure or association to other molecules, resulting in complete degradation when the membrane is solublized.

Thirdly, treatment of CNP2 expressing cells with kinase activators results in non-mitochondrial localization of CNP. Import of proteins into mitochondria is not a reversible process since the N-terminal targeting sequence is usually cleaved, resulting in a protein different in its primary structure than its precursor. Rather, it is likely that N2 of CNP2 is involved in the binding/association to a mitochondrial element, which is disrupted by phosphorylation.

Lastly, mitochondrial localization is largely affected by the isoprenylation, as demonstrated by the substitution of cysteine 417 to serine. Elimination of the cysteine 417, which is a target of isoprenylation, causes a dramatic shift in the number of cells showing mitochondrially localized CNP2. A decreased number of cells still display mitochondrial localization of CNP2, which indicates that both the N-terminal sequence of CNP2 and isoprenyl group are involved in mitochondrial localization, where N-terminal sequence is an essential targeting sequence and the isoprenyl group subsequently stabilizes this localization. The mechanism of mitochondrial localization of CNP2 has not been determined. However, it is likely that the isoprenyl group assists in the localization by anchoring the protein into the outer membrane of mitochondria. In such a scenario, import of a protein which is anchored by the isoprenyl group, is not likely to occur.

Phosphorylation of CNP2 is a crucial regulator of CNP2 localization. It was proposed that the negative charge introduced into the N2 of CNP2 by phosphorylation negates the overall positive charge, which is a characteristic of a mitochondrial import signal. It is interesting to note that there is another protein with its mitochondrial import regulated by phosphorylation. An isoform of glutathione S-transferase (GST) is found to be imported into mitochondria when residues of its import signal at the

C-terminus are phosphorylated (Robin et. al., 2003). Phosphorylation of this protein is found to increase interaction with a chaperone, which enhances the mitochondrial targeting. Substitution of serine 9 and 22 of CNP2 to glutamate mimics the phosphate group by introducing the equivalent negative charge. However, the validity of these substitutions as a replacement of phosphate is questionable. First, it is not known how the phosphorylation exerts its effect on mitochondrial targeting. The import signal sequence may be the only factor involved in the targeting, or it may interact with other chaperones as previously described. Introduction of negative charge effectively negates the overall positive charge of the import signal. However, if the phosphate groups exert its effect through other mechanisms, the substitution of serine residues by glutamate is not an accurate model for phosphorylation, as the side chain of glutamate does not accurately reflect the structure of the phosphate group. Secondly, CNP is likely to be associated with mitochondria by means other than import. In such a case, the N-terminal sequence of CNP2 is not an import signal, and its phosphorylation functions through a mechanism different from the proposed one. It is likely that phosphorylation disrupts association of CNP2 with mitochondrial proteins, or other molecules exposed on the outer membrane of mitochondria.

There are hints of CNP2 phosphorylation by both PKA and PKC. Initial experiments with PKA and PKC activators suggested that PKC may be the activator, and previous results by O'Neill suggested that serine 9 was phosphorylated in the presence of Phorbol dibutyrate (PDB), an activator of PKC. However, a time course with both activators shows that activation of PKA by dbcAMP yields an almost equal effect on mitochondrial localization of CNP2, and the effect gradually diminishes after 1 hour. Signaling pathways are regulated tightly to adjust to the external conditions, and artificial activation such as use of chemical activators is eventually down-regulated. Hence it is understandable that the effect of dbcAMP is not seen in cells treated overnight. Several experiments were conducted by another member of our lab to identify the kinase involved in phosphorylation of CNP2 (Lee, unpublished data). Various CNP constructs were expressed in E. Coli, and purified proteins were subjected to in-vitro phosphorylation by commercially available kinases in the presence of radioactive phosphate. Each serine residue was tested using the same mutants (S9A, S22A, S9, 22A) as previously described. CNP1 was used as a control to identify phosphorylation unique to CNP2. In-vitro phosphorylation of CNP2 by PKA shows preferential phosphorylation of serine 22, followed by much weaker phosphorylation on serine 9. Comparisons to the consensus sequence of PKA-phosphorylated serine

reveal that serine 22 is an ideal phosphorylation site, whereas serine 9 shows slight deviation. On the other hand, preliminary results suggest that PKC preferentially phosphorylates serine 9, as well as serine 22 and other unidentified residue(s) within the main sequence of CNP, resulting in phosphorylation of CNP1. Work is still in progress regarding in-vitro phosphorylation of CNP2 by PKC. Numerous observations of CNP phosphorylations are published, and both PKA and PKC are candidate kinases. Despite preferences by each kinase towards a particular serine residue, activation of one kinase was enough to target CNP away from mitochondria. This shows that both kinases are able to successfully phosphorylate serine 9 and 22, given enough activation. Also, in-vitro phosphorylation of CNP2 resulted in phosphorylation of both residues by either kinase (Lee, unpublished data). However, it is likely that under physiological conditions where kinases are not hyper-activated by presence of excessive activators, specificities of PKA and PKC become a significant factor, and it requires a coordination of both kinases in order to phosphorylate the two serine residues within the CNP2 unique region.

Despite the effort to draw conclusion from the collected data, the outcome is rather ambiguous. Numerous problems were encountered during many of the experiments. One is that the method of transfection used in the experiments yields

very low transfection rates. Maximum transfection achieved using HeLa S3 is less than 10%. HeLa S3 is known to have poor transfection efficiency, but this was the only cell line where mitochondrial localization of CNP2 is well characterized. Other cell lines, such as COS-7, do not show any mitochondrial localization of CNP2, although the transfection rate is much higher. Due to this low transfection rate, it was inevitable that procedures such as immunoprecipitation would be marred by the presence of many contaminating bands. To address this issue, various other methods of transfection were attempted and a viral vector was constructed. However, all transfections resulted in low efficiency, and the viral vector was not completed in time for proper experimentation. Electron microscopy of transfected cells were attempted as well. However, due to low expression of CNP, no conclusion could be reached from these experiments.

The function of CNP is still not certain. However, it is well characterized that CNP interacts with microtubules. Also, there is a possibility of GTPase activity by CNP, which is currently under investigation. The role of CNP2 isoform as it relates to the localization to mitochondria can be extrapolated from the known facts as follows. First, CNP2 may be involved in the transport of mitochondria via interaction with cytoskeletal elements. Subcellular organelles have strict organization, and they are not

free floating in the cytoplasm. Extensive cytoskeletal networks regulate the movement and location of each organelle, and mitochondria are not an exception. Mitochondria of myelinating cells are strategically placed to perform specific functions, such as monitoring of local calcium levels (Haak et. al, 2000). Since mitochondria are known to associate with microtubules for the transport and localization (Panesse et. al., 1996), it is possible that CNP2 may be an anchor that provides the site of attachment on mitochondria for cytoskeletal network for the locomotive forces.

Secondly, CNP2 may be involved in the splicing of t-RNA. It has been reported that t-RNA splicing endonuclease is localized to mitochondria in yeast. GTPase activity is required in the event of t-RNA splicing. This may be provided by CNP2 if both CNP2 and t-RNA splicing endonuclease are located on the outer membrane of mitochondria. The catalytic domain of CNP possesses aromatic and positively charged residues on β-sheet structure, which is typical of a common RNA-binding motif (Kozlov et. al., 2003).

Chapter 4: General Discussion

Recent developments regarding CNP has provided some very useful information towards understanding its physiological role. Keeping these in mind, the results presented in this thesis will be briefly summarized and further discussed in the following paragraphs.

From the results obtained in these experiments, the possibility of CNP and Fyn interaction is unlikely. However, involvement of CNP in signaling cascade can not be ignored. CNP is found to be phosphorylated on three or more residues, two of them being identified as serine 9 and 22 of the longer CNP2 isoform. This leaves one or more possible phosphorylation site(s) within the CNP1 sequence. Taken together with the finding that CNP is found in lipid rafts, it still remains a strong possibility that CNP is involved in a signaling cascade leading to myelination. Aside from Src family, common kinases found in lipid rafts includes not only tyrosine kinases such as Lyn (Kovárová et. Al., 2001), PI-3 kinase (Parpal et. Al., 2001) and MAP kinase (Wu et. Al., 2002), but also common serine/threonine kinases such as PKC (Dienz et. Al., 2003). Some of these kinases are known to be active during the initial stages of myelination. It is well documented that interaction of CNP with cytoskeletal elements bring morphological changes that transforms cells into oligodendrocyte-like shape (Lee, unpublished data). Thus, phosphorylation of CNP may be the activator initiating

changes in cytoskeletal arrangements, leading to membraneous extensions of oligodendrocytes resulting in myelination.

Function of CNP in non-neural tissues is even more obscure. However, its association with mitochondria suggests a possible function without tissue specificity. On the other hand, CNP expression is found only in a limited number of places. How mitochondria in these tissues differ is not known. Also, it is uncertain whether CNP in these tissues are serving their role in mitochondria or in other subcellular locations. So far, only adrenal chromaffin cells are proven to show CNP localized to mitochondria. From current knowledge regarding CNP in non-neural tissues, the following possibilities can be considered. First, CNP serves a role of transport by means of binding different organelles and molecules. As CNP is isoprenylated, its association with membraneous structures other than the cell membrane and mitochondria is certainly a possibility. Also, binding of CNP with RNA (Gravel, unpublished data) suggests another possibility of transport within a cell.

Secondly, CNP may be serving a function which is needed in membrane-rich structures, as presence in myelin and mitochondria indicates. A common feature of myelin and mitochondria is that they are rich in lipid membrane, and show very

organized and defined morphology. Presence of CNP in two membrane-rich structures may not be a coincidence. Another possible non-neuronal function of CNP is involvement in apoptosis. In a rough preliminary experiment, where HeLa cells were stained for endogenous CNP, elevated signal was observed in cells in which mitochondrial morphology resembled a state of apoptosis. Further investigations were not carried out, therefore it remains rather a far-fetched prediction. However, it is an interesting possibility worth a brief investigation.

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