2007

Examination of Post-Translational Modifications and Associate Proteins of PCBP1 in Neuronal Cells

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Examination of Post-translational Modifications and Associate Proteins of PCBP1 in Neuronal Cells

Ying-Chih Lin

Submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biology from the Department of Biology of Seton Hall University December 2007
Abstract

Poly C binding protein 1 (PCBP1) is known to participate in the regulation of 𝜇-opioid receptor (MOR) gene expression in neuronal cells. Our recent study showed that during mitosis PCBP1 appeared in the cytosol but not on chromosomes. We therefore investigated the expressed protein patterns of PCBP1 in neuronal cells during mitotic and non-mitotic phases and its possible post-translational modifications (PTMs).

First, the expression pattern of PCBP1 was examined using SDS-PAGE and Western blot analysis. The result revealed multiple forms of PCBP1 including one distinct form with MW above 66 kDa found exclusively in mitosis, doublet bands around 38 kDa, and two sets of protein ladders at 38-66 kDa and ~97 kDa in both mitotic and non-mitotic cells. Interestingly, the doublet form of PCBP1 in mitosis demonstrated a slower migration rate than that in non-mitotic cells. These findings suggested the occurrence of PTM on PCBP1. Using immunoprecipitation and then Western blot analysis, we further determined no endogenous acetylated or phosphorylated PCBP1 was found in vivo using the anti-acetylation or phosphorylation antibodies.

The addition of NEM in cell lysate induced a mobility change of the doublet PCBP1 at 38 kDa compared to that in non-NEM treated lysate under mitotic and non-mitotic conditions. Since NEM is known for inhibiting desumoylation/ deubiquitinating enzymes, this result indicated that PCBP1 may be modified through a NEM-related PTM such as sumoylation or ubiquitination. Further exploring the probability of sumoylation, the parallel immunoblots of PCBP1, SUMO-1, -2 and -3 were compared. One band close to 220 kDa in both mitotic and non-mitotic cells and the prominent form above 66 kDa in
mitosis appeared to be modified by SUMO-1. Two bands of PCBP1 within the protein ladder at 38-66 kDa were indicated as SUMO-3 conjugates while one form of PCBP1 was implied to be attached by SUMO-2. In summary, PCBP1 may be a substrate for sumoylation in neuronal cells.

In addition to the study on PTM regulation of PCBP1 in neuronal cells, we also examined whether PCBP1 associates with Sp factors, since PCBP1 demonstrates functional interplay with these factors in modulating MOR gene expression. Using coimmunoprecipitation and Western blot, our results displayed that PCBP1 physically interacts with Sp3 in a non-mitotic state, but not with Sp1 in either mitotic or non-mitotic conditions. Further investigation of PTMs on PCBP1 and how PCBP1 associates with Sp3 may grant greater understanding of its versatile roles in neuronal cells.
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Acknowledgements

I would like to express my sincerest gratitude to my mentor, Dr. Jane Ko, for her guidance and enduring patience throughout the course of my research.

I also wish to thank Dr. Allan Blake for his support and for letting me conduct somatostatin-related experiments during my first semester in Seton Hall. He therefore serves as my co-mentor.

I would also like to thank my committee members, Dr. Carroll Rawn and Dr. Angela Klaus, for the long hours they spent reviewing and giving valuable feedback on my work.

Additional thanks go to the members of Dr. Ko’s lab, especially Kelly Flock and Hamidah Sultan for their everyday support and companionship during my time at Seton Hall. Special thanks to Andrea Berry for the transfection and confocal experimental data in Figure 1.

Thanks also to Dr. Carolyn Bentivenga for giving me the opportunity to pursue my Master’s degree at Seton Hall and the members of the entire Biology Department for their continued support.

Finally, I wish to thank my family in Taiwan and friends throughout the United States for their encouragement and support.
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Abstract

Poly C binding protein 1 (PCBP1) is known to participate in the regulation of μ-opioid receptor (MOR) gene expression in neuronal cells. Our recent study showed that during mitosis PCBP1 appeared in the cytosol but not on chromosomes. We therefore investigated the expressed protein patterns of PCBP1 in neuronal cells during mitotic and non-mitotic phases and its possible post-translational modifications (PTMs).

First, the expression pattern of PCBP1 was examined using SDS-PAGE and Western blot analysis. The result revealed multiple forms of PCBP1 including one distinct form with MW above 66 kDa found exclusively in mitosis, doublet bands around 38 kDa, and two sets of protein ladders at 38-66 kDa and ~97 kDa in both mitotic and non-mitotic cells. Interestingly, the doublet form of PCBP1 in mitosis demonstrated a slower migration rate than that in non-mitotic cells. These findings suggested the occurrence of PTM on PCBP1. Using immunoprecipitation and then Western blot analysis, we further determined no endogenous acetylated or phosphorylated PCBP1 was found in vivo using the anti-acetylation or phosphorylation antibodies.

The addition of NEM in cell lysate induced a mobility change of the doublet PCBP1 at 38 kDa compared to that in non-NEM treated lysate under mitotic and non-mitotic conditions. Since NEM is known for inhibiting desumoylation/ deubiquitinating enzymes, this result indicated that PCBP1 may be modified through a NEM-related PTM such as sumoylation or ubiquitination. Further exploring the probability of sumoylation, the parallel immunoblots of PCBP1, SUMO-1, -2 and -3 were compared. One band close to 220 kDa in both mitotic and non-mitotic cells and the prominent form above 66 kDa in
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In addition to the study on PTM regulation of PCBP1 in neuronal cells, we also examined whether PCBP1 associates with Sp factors, since PCBP1 demonstrates functional interplay with these factors in modulating MOR gene expression. Using coimmunoprecipitation and Western blot, our results displayed that PCBP1 physically interacts with Sp3 in a non-mitotic state, but not with Sp1 in either mitotic or non-mitotic conditions. Further investigation of PTMs on PCBP1 and how PCBP1 associates with Sp3 may grant greater understanding of its versatile roles in neuronal cells.
**Introduction**

Opioids, such as naturally-occurring morphine, are chemical substances used mainly for pain relief via binding to the opioid receptors. The mu opioid receptor (MOR), found throughout the central nervous system, is the key player in mediating the morphine-induced analgesic effect as well as the effects of tolerance and dependence (Kieffer and Evans 2002; Sora et al., 1997). It has been reported that transcription of the MOR gene in mouse brains is primarily initiated via its proximal promoter (Ko et al., 1997). Further studies have suggested that several transcription factors participate in the regulation of mouse MOR gene expression by interacting with its proximal promoter (Ko et al., 1998; Ko and Loh, 2005).

Two *cis*-acting elements in the proximal promoter were found to be critical for its activity: an inverted-GA (iGA) motif bound by Sp1 and Sp3 and a canonical Sp1 binding site. Binding of Sp transcriptional factors to both regions simultaneously can activate transcription of MOR in an additive manner (Ko et al., 1998). Sp1 and Sp3 are members of the Sp family that share a conserved DNA-binding domain in the C-terminus. They feature three zinc fingers of the Cys2-His2 class that interact with the GC box found in the promoters of many genes (Spengler et al., 2005).

In addition to the double-stranded (ds) Sp factor binding sites on the MOR proximal promoter, a single-stranded (ss) DNA polypyrimidine (PPy) region, was found to be essential for the core proximal promoter activity as well (Ko and Loh, 2001). By using the yeast one-hybrid screening system, poly C binding protein 1 (PCBP1) was identified as a ssDNA binding protein which binds to the ss polypyrimidine (PPy) element of the
MOR proximal promoter and participates in neuronal MOR gene expression (Ko and Loh, 2005).

**KH domain superfamily & PCBP 1**

The poly C binding proteins (PCBPs) belong to the KH domain superfamily which is characterized by specific binding to both RNA and DNA. There are five evolutionarily related KH domain-containing proteins in mammalian cells: hnRNPs K and αCP1-4, also known as PCBP1-4. The KH domain, originally identified in hnRNP K as a RNA-binding motif, also recognizes ss structure in DNA. These five proteins share an overall structure consisting of two KH domains at the N-terminus and a third KH domain closer to the C-terminus. The variable region between the second and third KH domains confers the greatest divergence among these members. PCBPs are defined both by their KH structure and by their poly C binding specificity to both RNA and DNA (Ostareck-Lederer et al., 1998; Makeyev and Liebhaber, 2002; Ko and Loh, 2005; Malik et al., 2006).

Members of the KH domain superfamily have been shown to be involved in the processing of pre-mRNA (Expert-Bezançon et al., 2002), shuttling of mRNA between the nucleus and the cytoplasm (Michael et al., 1997), mRNA stabilization (Kiledjian et al., 1995; Wang et al., 1995; Chkheidze et al., 1999; Holcik and Liebhaber, 1997), translational activation (Blyn et al., 1997; Gamarnik and Andino, 1997) and silencing (Ostareck et al., 1997; Collier et al., 1998), as well as transcriptional regulation.
(Michelotti et al., 1996; Tomonaga and Levens, 1996; Ko and Loh, 2005; Kim et al., 2005; Meng et al., 2007).

The RNA-binding activity of PCBP1 has been studied in great detail. For example, its binding to mRNA 3' untranslated region (UTR) poly C rich elements may represent a general mechanism in stabilization of mRNA such as human \( \alpha \)-globin mRNA (Chkheidze et al., 1999), hypoxia-induced tyrosine hydroxylase (TH) mRNA (Paulding and Czyzyk-Krzeska, 1999), and erythropoietin (EPO) mRNA (Czyzyk-Krzeska and Bendixen, 1999). Translational silencing of erythroid 15-lipoxygenase (LOX) mRNA is mediated by PCBP1 and hnRNP K binding to the differentiation control element (DICE) in the 3' UTR during early erythroid differentiation, thereby preventing the 60S ribosomal subunit from joining to the 40S complex at the initiation codon (Ostareck et al., 1997, 2001). In contrast, the role of PCBP1 in translational enhancement has also been reported. PCBP1, together with PCBP2, functions as a translational coactivator of poliovirus RNA through a sequence-specific interaction with stem-loop IV of the internal ribosome entry site (IRES), resulting in cap-independent translation. Both PCBP1 and PCBP2 can interact with the poliovirus RNA 5'-terminal cloverleaf structure and stimulate 3CD binding which is a precursor of viral protease-polymerase to promote poliovirus RNA replication (Ostareck-Lederer et al., 1998; Gamarnik and Andino, 1997).

In addition to its functions regulating certain genes at the post-transcriptional and translational levels, PCBP1 has been shown to be a ssDNA-binding protein participating in neuronal MOR gene expression at the transcriptional level (Ko and Loh, 2005). PCBP1 can be recruited simultaneously with Sp1 and Sp3 factors to the MOR proximal
promoter *in vivo* and enhance the trans-activation of Sp1 and Sp3 on the proximal promoter of MOR respectively in an additive manner (Rivera-Gines et al., 2006). Further analysis has revealed that the molecular basis of transactivation on MOR gene proximal promoter by PCBPI is conferred by the cooperation of the three sequential domains of PCBPI, although the single KH domain possesses the MOR ssDNA binding ability (Malik et al., 2006).

Recently, PCBPI has been reported to be present throughout the cytoplasm and nucleus in neuronal cells, with a preferential nuclear expression. The data also suggests that a correlation exists between the nuclear punctate pattern of PCBPI in neuronal cells and its ssDNA binding ability at its target sites on chromatin (Berry et al., 2006). Furthermore, our laboratory recently found that during mitosis PCBPI is present solely in the cytoplasm, but not on the chromosomal DNA by using the confocal microscopy analysis (Figure 1) with EGFP-PCBP1 transfected neuronal cells. It has been shown that Runx2, a transcription factor, is stable and associated with chromosomes during mitosis (Young et al., 2007). These observations implicated that there may be posttranslational modifications (PTMs) on PCBPI which result in the change of its ssDNA binding ability and cellular distribution during mitosis.
**Figure 1.** The cellular distribution of PCBP1 in neuronal mitosis cell. Neuro2A cells transfected with EGFP-PCBP1 plasmid were viewed under a laser scanning confocal microscope. PCBP: the EGFP-fused PCBP1 indicated by green color appeared in the cytosol. PI: the red color represents chromosomal DNA structure stained by propidium iodide. Merge: the merged image of PCBP and PI, showing there is no co-localization (supposed to be yellow color) of the green fluorescence labeled PCBP1 and PI-stained chromosome.
Post-Translational Modifications (PTMs)

Protein functions can be tightly controlled by a variety of mechanisms at the translational and post-translational levels. One of the predominant mechanisms cells employ to regulate multifunctional proteins is posttranslational modification (PTM). There are more than 300 different PTMs such as proteolysis, phosphorylation, glycosylation, methylation, acetylation, ubiquitination, and sumoylation. PTMs may change proteins’ function by altering their size, charge, structure, and functional group, which may result in the change of cell compartmentalization, sequestration, degradation, and protein-protein interaction. Individual proteins can undergo multiple and different PTMs (Clark et al., 2005). Not surprisingly, malfunctions in these critical cellular processes are usually considered to be a cause for many illnesses. Comprehension of the physiological relevance of these modifications is extremely important, as it may lead to the discovery of new pharmacological targets that could provide therapies for human diseases or illness.

Acetylation

Acetylation of lysine residues within proteins has been revealed to be one of the PTMs used by cells for regulation of gene transcription. Acetylation of transcription factors and histones can regulate protein-DNA and protein-protein interactions. Histones are acetylated on lysine residues in the N-terminal tail to disrupt the nucleosome-nucleosome contacts, resulting in the activation of gene transcription; this disruption can later be reversed by deacetylation of the histone tails (Bannister and Miska, 2000). These
reactions are catalyzed by enzymes with histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity. Transcription coactivators, such as CBP/p300, PCAF, and SRC-1 possess intrinsic HAT activity which may function via their modification on histones or non-histone proteins (Ogryzko et al., 1996; Spencer et al., 1997; Bannister and Miska, 2000).

Site-specific DNA binding factors are one of the categories of non-histone substrates of acetyltransferase directly involved in transcription. For instance, acetylation of p53 results in an increase of its DNA binding ability (Gu and Roeder, 1997; Bannister and Miska, 2000) and acetylation of lysine residues in the inhibitory domain of Sp3 causes silencing of its transcriptional activity (Braun et al., 2001). In addition, p53 is also known for controlling neuronal apoptosis (Slack et al., 1996), and recently it has been demonstrated to promote neurite outgrowth and axon regeneration after nerve injury when the lysine 320 of p53 is acetylated (Di-Giovanni et al., 2006). Proteins not directly related to transcription activity can be acetylated by transcription coactivators as well, e.g. the acetylation of the nuclear import factor Rch1 by CBP/p300 (Bannister and Miska, 2000).

**Phosphorylation**

The most common of PTMs is protein phosphorylation, which is capable of changing a protein’s function and conformation by adding phosphate group to serine, threonine, or tyrosine residues via protein kinases. More than 1000 protein kinases are predicted in human genome. Phosphorylation/dephosphorylation has substantial
influences on various cellular functions such as metabolic pathways, gene expression, cell division, signal transduction, or apoptosis (Clark et al., 2005; Gomperts et al., 2004). For example, phosphorylation of hnRNP K can increase the association with its protein partners such as Lck and proto-oncoprotein Vav, while decreasing its DNA and RNA-binding activity (Makeyev and Liebhaber, 2002). The c-Src kinase can also phosphorylate tyrosine 458 of hnRNP K, which results in the inhibition of its binding to the DICE element in the 3’ UTR and impairs its ability to inhibit LOX mRNA translation (Messias et al., 2006).

**Sumoylation**

Sumoylation is an emerging PTM in which small ubiquitin-like modifiers (SUMO) covalently conjugate to target proteins via the formation of an isopeptide bond between their C-terminal glycine and the ε-amino group of lysine residues in a substrate. Among the three mammalian SUMO isoforms (SUMO-1, 2 and 3), SUMO-2 and -3 are very similar in sequence, forming a subfamily that is distinct from SUMO-1. The SUMO conjugation mechanism is initiated with the cleavage of a SUMO precursor by specific hydrolases to become a mature protein. The mature SUMO protein is then activated by binding to the E1 activating enzyme and transferred to the E2 conjugating enzyme before being bound to the target protein under E3 ligase’s assistance. Only a small proportion of SUMO target proteins remain sumoylated at steady state *in vivo*, due to the presence of cysteine protease (SUMO isopeptidase).
SUMO modification has been suggested to be involved in diverse biological processes such as cell cycle progression, transcription and nuclear trafficking, and it may be associated with diseases ranging from neurodegeneration, diabetes to inflammation (Hay 2005; Bossis and Melchior, 2006). For example, SUMO-1 conjugation to RanGAP-1 and IκBα can regulate their nuclear transports and protein stabilities (Matunis et al., 1996; Desterro et al., 1998). Sumoylation of topoisomerase II by SUMO-2/-3 isoforms in mitosis has been suggested to be associated with chromosomal segregation (Azuma et al., 2003).

**Goal**

Since PTMs are common mechanisms for altering protein functions, in this research we examined if (1) PCBP1 is modified by PTMs in neuronal cells, and (2) PCBP1 physically interacts with Sp factors in neuronal cells.
Materials and Methods

Cell culture

Human neuroblastoma NMB cells and SH-SH5Y cells were maintained in Roswell Park Memorial Institute Medium (RPMI) supplemented with 10% heat inactivated Fetal Calf Serum at 37°C in a 5% CO₂ incubator.

Nocodazole treatment and preparation of cell lysates

NMB cells were synchronized using serum-free media and then treated with nocodazole for 24 hr to arrest cells in the mitotic phase (Hoebeke et al. 1976; Spyer and Allday 2006), or treated with vehicle, dimethyl sulfoxide (DMSO), as the control. The mitotic cells were collected by mechanical shake-off. Lysates of NMB, SH-SH5Y cells and rat brain were prepared using the lysis buffer, containing 1% SDS in Phosphate Buffer Saline (PBS) with various protease inhibitors including phenylmethylsulphonyl fluoride (PMSF), Na₃VO₄, pepstatin, and aprotinin.

TSA treatment

NMB cells treated with alcohol (vehicle control) or deacetylase inhibitor TSA (4,6-dimethyl-7-[p-dimethyaminophenyl]-7-oxohepta-2,4 dienohydro-xamic acid, Sigma, St. Louis, MO) for 24 hr were used as the positive control for the acetylation assay.
Cell fixation and staining

Cells grown on coverslips were fixed with 4% paraformaldehyde in PBS, and then perforated with 0.1% Triton X-100. Cells washed with PBS were stained with DAPI (4', 6-diamidino-2-phenylindole), and mounted onto slides with a drop of anti-fade reagent (Vector Labs, Burlingame, CA).

Lowry Assay

Protein concentration was determined by Lowry assay using bovine serum albumin as the standard. Samples first incubated with Solution C [1.8mM Na₂CO₃, 98mM NaOH, 0.95mM sodium potassium tartarate, and 0.4mM CuSO₄ 5H₂O] at room temperature (R.T.) were then further incubated with 1N Folin & Ciocalteu’s Phenol reagent. The protein concentration was determined by colorimetry at 660 nm.

Western blotting

Samples were separated on 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then electrotransferred to phenylmethanesulfonyl fluoride (PVDF) membranes. The membrane was probed with a specific antibody, and signals were detected using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). The anti-PCBP1, p-Tyr, p-Thr HRP, Sp1 and Sp3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-SUMO-1, -2, and -3 antibodies were purchased from Invitrogen (Carlsbad, CA). The
anti-acetyl-Lys-antibody and another anti-SUMO-1 antibodies were purchased from Cell Signalining (Danvers, MA), while the anti-p-Ser antibody from Sigma (St. Louis, MO).

**Immunoprecipitation**

Protein-G agarose beads were added to cell lysates at $4^\circ$C overnight following the antibody incubation at $4^\circ$C for 2 hr. The beads were washed three times with washing buffer containing NP-40, SDS, and sodium deoxycholate in PBS to remove the non-specific binding proteins. The immunocomplex was eluted with SDS-PAGE treatment buffer.
Results

Acquiring human neuronal mitosis cells

In order to obtain neuronal mitotic cells for studying the potential PTMs of PCBP1 (Fig. 1), human NMB cells were synchronized by serum starvation. The synchronized cells were then arrested in a mitotic state using nocodazole, an inhibitor of microtubule polymerization. The control cells were treated with vehicle. DNA was stained with DAPI and viewed under a laser scanning confocal microscope. Chromosomal DNA in mitotic cells formed a rosette structure due to the condensation of DNA molecules (Fig. 2A), while an uncondensed DNA structure was observed in the non-mitotic control cells (Fig. 2B). Using this combination of starvation and nocodazole treatment, mitotic cells were obtained for further studies.
Figure 2. Comparison of stained mitotic and non-mitotic cell nuclei. DNA was stained with DAPI (blue) and then viewed using a confocal microscope. A) The chromosomal DNA featured a rosette structure in mitotic cells as a result of nocodazole treatment. B) Staining of nuclei in non-mitotic cells treated with vehicle.
Examination of endogenous PCBP1 expression pattern using Western blot analysis

Proteins from mitotic or control cell lysates were separated by 10% SDS-PAGE and then analyzed by Western blot using anti-PCBP1 antibody. In Fig. 3A left, multiple PCBP1 bands were present. One major protein with the molecular weight of approximately 38 kDa, as previously reported (Ko and Loh, 2005), was observed in both mitotic and control cell samples. The other prominent band above 66 kDa was also found exclusively in mitotic cells. In addition, multiple bands between 38 and 66 kDa as well as above 97 kDa were also detected. To further examine if these bands were specific recognized by the anti-PCBP1 antibody, a peptide competition assay was performed. The results in Fig. 3A right demonstrated that these bands were specific binding by PCBP1 antibody.

To further examine if 38 kDa protein bands showed any difference between mitotic and control cells, a 12% SDS-PAGE was used. In Fig. 3B, the 38 kDa band was resolved into two separated protein bands, and the doublet bands migrated slightly slower in mitosis than those in the control group, suggesting that 38 kDa PCBP1 displayed differential gel mobility between mitotic and control cells.

Taken together, data demonstrated the presence of multiple forms of PCBP1 in neuronal cells, in addition to the unmodified 38 kDa form. These results also implied that various PTMs of PCBP1 may take place, with a specific PTM of PCBP1 occurring solely during mitosis.
Figure 3. Comparison of PCBP1 electrophoretic mobility. Protein extracts from mitotic (M) and control (C) cells were analyzed by SDS-PAGE and Western blotting with anti-PCBP1 antibody. A) Expression pattern of PCBP1 in neuronal cells was detected by anti-PCBP1 antibody (left) and anti-PCBP1 antibody with PCBP1 peptide (right). B) The doublet PCBP1 around 38 kDa is observed in both mitotic and control cells. Protein bands with M.W. around 38 kDa are indicated by (→) and (←). A protein band with M.W. above 66 kDa is indicated by (*). Multiple proteins between 38-66 kDa is indicated by ([]) and the protein bands with M.W. above ~97 kDa is marked by ({}).
Examination of acetylation on endogenous PCBP1 lysine residues

To determine if any PTM contributes to multiple PCBP1 protein bands observed in Western blot analysis, we first examined whether acetylation modification occurred in vivo. Acetylation of transcription factors plays a critical role in regulating gene expression. For example, p53 tumor suppressor is activated in response to DNA damage via an acetylation mechanism (Ito et al., 2001).

PCBP1 from neuronal cell lysates was immunoprecipitated using anti-PCBP1 antibody. The immunoprecipitates were then analyzed using an anti-acetyl-lysine antibody. In Fig. 4A, no significant acetylated protein band was detected in either mitotic or control cell lysates. However, the positive control sample, treated with TSA to increase acetylation on p53, contained the acetylated protein band. To ensure the success of the immunoprecipitation (IP) process, the same blot was then probed with anti-PCBP1 antibody. In Fig. 4B, the immunoprecipitates did contain the PCBP1 doublet bands around 38 kDa, which also showed the differential gel mobility between mitotic and control cells as in Fig. 3B. Thus, these results suggested that PCBP1 was not acetylated in mitosis or control cells.
**Figure 4.** Examination of acetylation on PCBP1 lysine residues *in vivo*. Cell lysates from mitotic (M) and control (C) cells were immunoprecipitated with anti-PCBP1 antibody, and then analyzed by SDS-PAGE and western blotting with (A) anti-acetyl lysine antibody, and (B) with anti-PCBP1 antibody. PCBP1 doublets around 38 kDa are indicated by (→) in (B). TSA treated cell lysate was loaded on the right lane as a positive control suggested by the manufacturer (Post Ctrl).
Examination of phosphorylation on endogenous PCBP1

Phosphorylation is a common PTM in regulating multifunctional proteins. In order to determine if PCBP1 is phosphorylated \textit{in vivo}, immunoprecipitates of endogenous PCBP1 from mitotic and control cell extracts were subjected to SDS-PAGE and Western blotting using three different anti-phosphorylation antibodies to identify the possible phosphorylated residues.

(1) Phosphorylation on serine residues

Cell lysates from mitotic (M) and vehicle treated (C) cells were immunoprecipitated with anti-PCBP1 antibody, and then the immunoprecipitates were analyzed by SDS-PAGE and Western blotting with anti-serine-phosphorylation antibody. In Fig. 5A, no significant PCBP1 band was observed in either mitotic or control cells. However, the rat brain served as a positive control did show a band recognized by the anti-serine-phosphorylation antibody. Further confirming the successful immunoprecipitation process, the same blot was then probed with anti-PCBP1 antibody (Fig 5B), and the presence of doublet bands of PCBP1 with a slower migration rate in mitotic cells than those in control cells was still observed. These results suggested that PCBP1 may not be phosphorylated on serine residues in mitotic or control cells.
**Figure 5.** Analysis of PCBP1 phosphorylation on serine residues *in vivo*. Mitotic (M) or vehicle-treated (C) cell lysates were immunoprecipitated with anti-PCBP1 antibody, and then examine using Western blot probing with A) anti-phospho-serine antibody and B) anti-PCBP1 antibody. PCBP1 doublet bands around 38 kDa are indicated by (→) in B). The positive control is lysates from the rat brain.
(2) Phosphorylation on tyrosine residues

In addition to serine residues, we explored the possibility of tyrosine phosphorylation on PCBP1 in neuronal cells using Western blot analysis with the anti-tyrosine-phosphorylation antibody. The cell lysates of SH-SH5Y was used as a positive control (Jope et al., 1999). In Fig. 6A, tyrosine-phosphorylated protein bands were observed in both SH-SH5Y and mitotic cells but not in control cells. The tyrosine-phosphorylated protein bands above 45 kDa were detected in mitotic cells. To confirm the successful immunoprecipitation process, the same blot was then probed with anti-PCBP1 antibody. Similar doublet PCBP1 bands around 38 kDa were observed in both mitotic and control cells (Fig. 6B). These results indicated that PCBP1 around 38 kDa was not phosphorylated on tyrosine residues in mitotic or control cells.
Figure 6. Examination of phosphorylation on tyrosine residues of PCBP1 in vivo. Mitotic (M) and control (C) cell lysates were immunoprecipitated with anti-PCBP1 antibody then analyzed by Western blot using A) anti-phospho-tyrosine antibody and B) anti-PCBP1 antibody. The doublet around 38 kDa PCBP1 is indicated by (→) in B). SH-SH5Y cell lysates is used as a positive control.
(3) Phosphorylation on threonine residues

The third common phosphate group acceptors are threonine residues; we then examined the possibility of threonine phosphorylation on the neuronal PCBP1 using immunoprecipitation with anti-PCBP1 antibody and Western blot analysis with the anti-threonine-phosphorylation antibody. In Fig. 7A, no significant threonine-phosphorylated protein bands were observed in mitotic or control cells. However, a strong threonine-phosphorylated protein band above 66 kDa was observed in the positive control, cell lysates from the same mitotic cells. The same blot was then probed with anti-PCBP1 antibody to verify the immunoprecipitation procedure. In Fig. 7B, a similar doublet form of PCBP1 around 38 kDa was detected in both mitotic and control cells. The PCBP1 band with MW above 66 kDa exhibited in a slower gel mobility as compared to that of the threonine-phosphorylated protein band. The data suggested that PCBP1 proteins with MW around 38 kDa or above 66 kDa are not phosphorylated at threonine residues in mitotic and control cells.

In summary, above data suggested that no significant phosphorylation of PCBP1 on serine, tyrosine or threonine residues was detected in mitotic or control cells.
Figure 7. Examination of phosphorylation on threonine residues of PCBP1 in vivo. Mitotic (M) or control (C) cell lysates were immunoprecipitated with anti-PCBP1 antibody then subjected to western blot probing with A) anti-phospho-threonine antibody and B) anti-PCBP1 antibody. The doublet form around 38 kDa is indicated by double arrows (→). The phosphor-threonine protein is indicated by an arrow (←) and the species above 66 kDa is marked by an asterisk (*). Mitotic cell lysates served as a positive control on the right lane.
*N*-ethylmaleimide induces an electrophoretic mobility change of endogenous PCBP1

To further investigate whether PCBP1 could be modified by SUMO conjugation due to the observation of protein ladders and the prominent band above 66 kDa (Fig. 3), mitotic and control cells were lysed and treated with cysteine protease inhibitor, *N*-ethylmaleimide (NEM), which has been shown to inhibit desumoylating enzymes and thereby stabilize sumoylated proteins since sumoylation is highly dynamic and usually under detection limit (Suzuki et al., 1999; Ross et al., 2002).

Lysates treated with or without NEM were analyzed using SDS-PAGE and Western blot with anti-PCBP1 antibody. As shown in Fig. 8, NEM treatment in mitotic cells resulted in slower-migrating PCBP1 bands around 38 kDa as compared to those without NEM treatment. In contrast, in mitotic cells the protein band with MW above 66 kDa was unaffected by the NEM treatment. The similar phenomenon with protein bands around 38kDa was also observed in control cell lysates treated with or without NEM.

These results suggested that NEM can change the migration rate of PCBP1 around 38 kDa in both control and mitotic cells, indicating that PTMs of PCBP1 were regulated through NEM-related pathways.
Figure 8. Analysis of NEM effect on PCBP1 gel mobility. Proteins from control and mitotic cell lysates treated with or without NEM were separated by SDS-PAGE and detected by Western blotting with anti-PCBP1 antibody. Multiple PCBP1 protein bands are indicated by (→), (*) and ([]).
Examination of Sumoylation on endogenous PCBPI

Above results indicated that PCBPI may be modified by sumoylation. We therefore examined the possibility of sumoylation using control or mitotic cell lysates in the presence of NEM, which inhibits the process of desumoylation. In Fig. 9A left, upon comparison of the parallel immunoblots against SUMO-1 and PCBPI antibodies, a faint PCBPI band with MW close to 220 kDa was recognized by anti-PCBP1 antibody, and the same position was also detected by anti-SUMO-1 antibody in mitotic and control cells. The same procedures were using the other SUMO-1 antibody from Invitrogen (Fig. 9A right). The corresponding position as the PCBPI form with MW above 66 kDa in mitotic cells on the PCBPI immunoblot was detected by anti-SUMO1 antibody in mitotic cell lysates.

SUMO-2 and -3 are the other possible sumoylation modifiers which can attach to protein substrates in mammalian cells. Comparing the SUMO-2, SUMO-3 and PCBPI immunoblots as shown in Fig. 9B, one band above 97 kDa in mitotic and control cells on SUMO-2 immunoblot migrated to the same position as one of the bands within the PCBPI ladder ~ 97 kDa. Meanwhile, Western blot using SUMO-3 antibody showed that two protein bands close to 45 and between 45 and 66 kDa moved to similar positions as the two multiple forms of PCBPI within 38-66 kDa.

Collectively, these data indicated that PCBPI may be modified by SUMO-1, -2 and -3 in mitotic and control cells.
Figure 9. Analysis of sumoylation on PCBP1 in vivo. Multiple forms of PCBP1 are indicated by (←→), (·), ([ ]), and (()). A) Left, cell lysates from control (C) and mitotic cells (M) were resolved on SDS-PAGE and probed with anti-SUMO1 and anti-PCBP1 antibodies respectively. Right, the same procedure was employed except using another commercially-made SUMO1 antibody. The corresponding SUMO-1 conjugated PCBP1 is marked by (←→) and (→). B) The same approach was applied as described in A), except using anti-SUMO-2, -3 and PCBP1 antibody. The corresponding SUMO-2, -3 conjugated PCBP1 is marked by (●) and (●→).
Association of Sp1, Sp3 with PCBP1 in vivo

Since PTMs may also result in the change of protein-protein interaction, we further examined if PCBP1 physically interacts with Sp1 and Sp3 (Sps), though PCBP1 and Sps can trans-activate the MOR gene proximal promoter in an additive manner (Rivera-Gines et al., 2006).

To determine whether Sp1 or Sp3 associates with PCBP1 in mitotic or control cells, the co-immunoprecipitation assay using anti-Sp1 or anti-Sp3 antibodies was performed. The immunoprecipitates were then subjected to SDS-PAGE and Western blot analysis using anti-PCBP1 antibody. The result shown in Fig. 10 revealed that Sp3 interacted with PCBP1 at 38 kDa in control cells but not in mitotic cells. The same blot was later probed with anti-Sp3 antibody to verify the successful immunoprecipitation procedure. However, no association between PCBP1 and Sp1 was observed using co-immunoprecipitation with Sp1 antibody (data not shown). Altogether, these results suggested that PCBP1 physically interacted with Sp3 only in the non-mitotic stage, but not with Sp1.
Figure 10. Analysis of protein-protein interaction of PCBP1 with Sp3. Mitotic (M) and control (C) cell lysates were immunoprecipitated with anti-Sp3 antibody or negative control goat serum (GS). The immunoprecipitates were subjected to immunoblotting with anti-PCBP1 or anti-Sp3 antibodies.
Discussion

Recently, differences in PCBPI subcellular localizations between mitotic and non-mitotic neuronal cells were observed. PCBPI is dispersed solely in the cytoplasm and not on DNA while cells are undergoing mitosis (Fig 1), while PCBPI appears in both the cytoplasm and nucleus in non-mitotic cells (Berry et al., 2006). The current study examined different expression patterns of endogenous PCBPI in mitotic and non-mitotic neuronal cells using SDS-PAGE and Western blot analysis. In addition to the unmodified 38 kDa protein, our results revealed multiple forms of endogenous PCBPI in neuronal cells (Fig 3): one prominent, higher molecular mass above 66 kDa present exclusively in mitotic cells, doublet 38 kDa forms, and two sets of protein ladders in the 38-66 kDa and ~97 kDa ranges. The doublet isoforms around 38 kDa in mitotic cells display slightly slower electrophoresis gel mobility than those in control cells. These results therefore suggested that PCBPI may be modified through post-translational modifications (PTMs).

The possibility of acetylation or phosphorylation modifications was first examined in this study. SDS-PAGE and Western blot analysis showed that no endogenous acetylated PCBPI was observed in neuronal cells (Fig. 4A), which was in agreement with the prediction of no potential acetylation sites within PCBPI using the NetAcet program (http://www.cbs.dtu.dk/services/NetAcet/). In addition, no phosphorylation on the endogenous PCBPI was identified using anti-phospho serine, tyrosine, and threonine antibodies in Western blot analysis (Figs. 5-7). Although no specific phosphorylation of PCBPI in neuronal cells was found using various anti-phosphorylation antibodies, our results can not eliminate the possibility for PCBPI to be phosphorylated upon exogenous
stimulations. It has been reported that phosphorylation on PCB1 threonine-60 and -127 residues occurs in HeLa cells by p21-activated kinase 1 (Pak 1) under EGF stimulation (Meng et al., 2007).

The other type of PTMs we investigated in this study were the conjugation of small protein or peptide groups, such as sumoylation, which can cause dramatic alterations in molecular weight and the appearance of protein ladders, as was observed in the expression pattern of PCB1 (Fig. 3). The addition of NEM in the lysis buffer induced a slower-migrating doublet in the 38 kDa region compared to that of the non-NEM treated lysate in both control and mitotic conditions (Fig. 8). The parallel comparison of the PCB1, SUMO-1, -2 and -3 immunoblots revealed that the band above 66 kDa in mitosis and one band close to 220 kDa in mitotic and control cells may be the SUMO-1 modified PCB1 (Fig. 9A); one band appears to be conjugated by SUMO-2 within the protein ladder of MW ~ 97 kDa, while two bands within the protein ladder in the 38-66 kDa range may represent the SUMO-3 modified PCB1 forms (Fig. 9B). The several forms of possible sumoylated PCB1 that were observed may be a result of monosumoylation at multiple sites (multisumoylation) or a sumo chain at a single site (polysumoylation) (Gocke et al., 2004; Tatham et al., 2001). It has been shown that NEM can stabilize SUMO-1 modified proteins, such as Sp1 and Sp3, in lysate (Spengler and Braittan, 2006; Ross et al., 2002). hnRNP K, a member of the KH domain family as PCB1, has been demonstrated to be a target for SUMO modification in vivo (Li et al., 2004). PCB1 appears to be a candidate substrate for sumoylation in neuronal cells and further validation is needed.
In addition to sumoylation, ubiquitination may be an alternative mechanism that can cause protein ladder formation and be affected by NEM. Ubiquitination is reversible by the activity of specific proteases known as deubiquitinating enzymes (DUBs) and NEM has been used as an inhibitor of endogenous ubiquitin isopeptidases (Ellison and Hochstrasser, 1991). Whether ubiquitination is the underlying mechanism of the NEM-induced electrophoresis mobility change of PCBPI requires future investigation.

The multifunctionality of PCBPI reflects its ability to interact with different protein partners. Using co-immunoprecipitation and Western blot analysis, PCBPI has been shown to interact with endogenous Sp3 in non-mitotic neuronal cells (Fig. 10), but not with Sp1 under either mitotic or non-mitotic conditions. These results indicate that the physical contact between Sp3 and PCBPI may be a part of the mechanism that allows additive transactivation of the MOR promoter (Rivera-Gines et al., 2006) under non-mitotic condition. However, PCBPI may not associate with Sp3 as a functional protein complex in mitotic cells. Furthermore, Sp3 simply immunoprecipitated the unmodified 38 kDa PCBPI instead of the other higher molecular weight forms, suggesting that the other modified PCBPI might possess different functions without associating with Sp3. The multiple forms of PCBPI with distinct PTMs may confer PCBPI different protein-protein interactions with corresponding functions. For example, tyrosine phosphorylation on hnRNP K in response to oxidative stress increases the association of hnRNP K with Lck and Vav (Ostrowski et al., 200). Thus, identification of associate proteins with PCBPI will be necessary to gain further insight into PCBPI's versatile functionality.
Conclusion

In summary, we have examined the possible post-translational modifications (PTMs) on PCBP1 and its association with the Sp factors in neuronal cells. The successfully obtaining mitotic cells using nocodazole treatment allowed us to determine the expression pattern of the endogenous PCBP1 under mitotic and non-mitotic conditions using SDS-PAGE and Western blot analysis. The presence of multiple PCBP1 forms in neuronal cells: one band above 66 kDa appearing exclusively in mitotic cells, one doublet around 38 kDa exhibiting differential gel mobility between mitosis and non-mitosis, and two sets of protein ladders in the 38-66 kDa and ~97 kDa ranges, suggesting the occurrence of PTMs.

Our data indicated that no acetylation or phosphorylation occurred on endogenous PCBP1 in mitotic or non-mitotic cells using immunoprecipitation and Western blot with anti-acetylation and various anti-phosphorylation antibodies. However, we do not rule out the possibility of phosphorylation being triggered by exogenous stimuli. The examination of sumoylation using NEM and SUMO-1, -2 and-3 antibodies suggested the possibility that PCBP1 may be a substrate for SUMO modification.

Furthermore, using co-immunoprecipitation and Western blot analysis, our result showed that Sp3 physically interacted with PCBP1 in non-mitotic cells, but not Sp1 under either cell conditions.

The mechanisms underlying PTMs of PCBP in neuronal cells and their corresponding physiological functions will be further studied.
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