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Investigation of the Protein-Protein Interaction Between PCBP1 and y -synuclein

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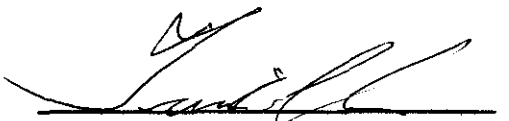

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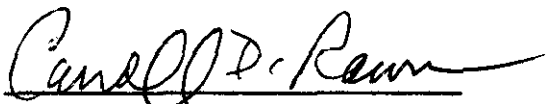

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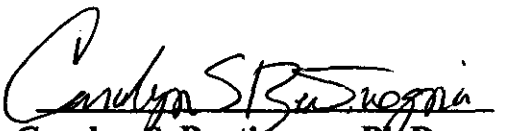

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Abstract

PCBP1 or poly (C) binding protein is a multifunctional protein, which belongs to the K homology domain superfamily. Its multiple functions include mRNA stabilization, translational regulation and transcriptional regulation, which have been found to affect the expression of the mu-opioid receptor gene. PCBP1's multiple functions may likely correlate to its various protein-protein interactions. We therefore examined its interacting protein(s) using a two-hybrid system by screening a cDNA library. After more than 1 million clones were screened, a candidate interacting protein was identified as γ-synuclein. The plasmid of this cDNA clone was purified and the interaction between PCBP1 and γ-synuclein was further confirmed using *in vivo* validation with the bacteria system as well as using GST-pull down assay.

To further examine if this physical interaction can take place in a mammalian cell system, several cell lines, including NMB (neuroblastoma), H292 (lung), HCT116 (colorectal), HT29 (colorectal), T47D (breast), and Mcf-7 (breast), were used. Western blot analysis showed that these human cancer cell lines endogenously expressed PCBP1 and γ-synuclein. The physical interaction of these two proteins was examined using cell lysates from these cell lines by co-Immunoprecipitation (co-IP) and subsequentially via Western blot analysis. Results suggested a physical interaction between endogenous PCBP1 and γ-synuclein. To further investigate if these cell lines expressed the identical γ-synuclein,

RT-PCR was performed using RNA from these cell lines with a γ-synuclein specific primer set. The PCR products were then subjected to DNA sequencing analysis.

The obtained DNA sequences were then compared with the published cDNA sequence in GenBank. This comparison showed a consistent missense mutation at the C-terminus of γ-synuclein. This mutation created a valine (non-polar) residue in the cells, whereas and a glutamic acid (acidic) residue in the database. A SNP database search was then performed to determine if this missense mutation was a SNP (single nucleotide polymorphism). A total of 53 SNPs were found for γ-synuclein and one of them matched the mutation found.

Since it is already known that PCBP1 participates in the regulation of the mu-opioid receptor gene expression, and based on this study that PCBP1 can physically interact with γ-synuclein, the effect of this protein-protein interaction on MOR promoter was further investigated using a luciferase reporter gene assay with the mammalian expression plasmid containing each form of γ-synuclein. Our preliminary results suggested that γ-synuclein may be able to modulate MOR promoter activity, which will need to be further confirmed. In conclusion, PCBP1 and γ-synuclein physically interact in the mammalian system and this interaction may modulate PCBP1's functional role.

Introduction

PCBP1 belongs to K Homology superfamily

The K homology superfamily contains 3 KH (K homology) domains, which are nucleic acid binding domains. They contain two KH domains near the N-terminus and a third one near the C-terminus. The greatest sequential variance among the family lies in the domain between the second KH domain and the third KH domain. This variable domain contains a NLS (nuclear localization signal), which gives this protein its ability to be shuttled between the nucleus and the cytoplasm (Berry et al., 2006). This superfamily contains two subcategories, the hnRNPs and the poly (C) binding proteins. PCBP1 is a member of the K homology domain superfamily, and is a protein approximately 38kDa in size. It is also known as αCP1 and hnRNP E1. It is an ssDNA and RNA binding protein which binds to poly cytosine residues. PCBP1 displays various physiological roles, including transcriptional regulation, translational regulation, and mRNA stabilization. It can form protein complexes and work in conjunction with other proteins to carry out various functions (Wang et al., 1999 and Rivera-Gines et al., 2006).

mRNA Stabilization

Binding of PCBP1 protein can cause mRNA stabilization of many genes. Alpha-globin mRNA is an example. PCBP1, PCBP2 and PABP (poly A binding protein) form an alpha complex which binds to a sequence in the 3'UTR of alpha-globin mRNA. The binding of this ribonucleoprotein complex prevents mRNA decay and prevents deadenylation (Wang et al., 1999). Another example is that PCBP1 along with hnRNP

A1 and hnRNP K bind to a sequence at the 3' UTR of collagen mRNA, and this binding stabilizes its mRNA and acts as a positive effector of synthesis (Thiele et al., 2004). A similar effect is also seen in renin and gastrin genes (Lee et al., 2007 and Adams et al., 2003). This increase in stability of mRNA and upregulation of protein synthesis caused by PCBP1 can also be seen for erythropoietin and tyrosine hydroxylase, but in a hypoxia dependent condition by binding to the 3' UTR of their mRNA and regulating stability (Zhu et al., 2002).

Transcriptional Regulation

Another function of PCBP1 is that it can serve as a transcriptional regulator. This function has been reported in two genes, eIF4E (eukaryotic translation initiation factor 4E) and MOR (mu-opioid receptor). PCBP1 binds to a specific sequence in the promoter region of eIF4E which contains eIF4E basal element (4EBE) and increases transcription (Meng et al., 2007). PCBP1 binds to a specific ssDNA sequence in the proximal promoter of mouse MOR gene and increases gene expression (Ko et al., 2005).

Translational Regulation

PCBP1 has also been reported as both a positive and negative translational regulator. The positive translational regulation has been reported in the c-myc gene. The proto-oncogene c-myc contains an internal ribosome entry segment (IRES) at its 5'UTR. PCBP1, PCBP2, and hnRNP K bind to c-myc's IRES mRNA and this binding leads to IRES mediated translation (Evans et al., 2003). Negative translational regulation has

been shown in the 15-lipoxygenase (LOX) gene. PCBP1 along with PCBP2 and hnRNP K form a complex and bind to the differential control element (DICE). The differential control element is a CU rich repeated motif at the 3'UTR. The binding of this protein complex prevents the 60S and 40S ribosome subunits from joining and forming the 80S ribosome at AUG sequence. Without the joining of the subunits, translation cannot occur (Ostarek et al., 1997). A similar effect is also reported in the A2 response element (A2RE), in which PCBP1 and hnRNPA2 together bind to A2RE. With this binding, the translation of A2RE RNA is prevented (Kosturko et al., 2006).

Other functions

PCBP1 has also been found to affect the cytoskeletal composition of neurons. PCBP1, PCBP2, and hnRNP K bind to the low, middle and high molecular mass neurofilament subunit proteins. These subunits will then co-polymerize and form neurofilaments (Thyagarajan et al, 2008). PCBP1 has also been found to act as a chaperone in the delivery of iron to ferritin, an iron storage protein (Shi et al., 2008).

To further understand PCBP1's different functional roles, our laboratory investigated this aspect by examining the protein-protein interactions using the two-hybrid system. This system employed two vectors, a bait protein containing vector and the other vector containing a cDNA library for screening candidate clone(s) of PCBP1 interacting protein. PCBP1 was used as the bait protein to screen its interacting protein(s) from a human brain cDNA library. Over 1 million clones were screened, and γ-synuclein was found to

be a candidate interacting protein. *In vivo* validation was performed using the purified plasmids via co-transformation. The confirmation of *in vitro* physical interaction was also obtained using the GST-pull down technique (Lab unpublished data).

Synuclein Family and γ-synuclein

The synuclein family contains α, β, and γ synuclein. The synuclein family has high amino acid sequence homology. Sequence comparisons show that α to β has 62% identity and 79% homology, α to γ has 50% identity and 74% homology, and β to γ has 47% identity and 66% homology. The highest divergence of amino acid sequence within the synuclein family is found in the C-terminus (Sung et al., 2006). These proteins have been shown to possess the ability to aggregate, and each member possesses different propensities for aggregation. The α-synuclein has the greatest propensity, and γ closely resembles it in its free state residual secondary structure. The β-synuclein possesses the least propensity for aggregation and it has been reported to protect α from aggregation in vitro (Sung et al., 2007). At the current stage, the functions of these three proteins have not been well understood.

The γ-synuclein is also known as breast cancer specific gene 1(BCSG1) and persyn, and is a small intracellular protein about 13kDa in size (Buchman et al., 1998). It locates at chromosome 10q23, and contains 5 exons coding for 127 amino acids (Lavedan et al., 1998). It was originally found in the nervous system and considered to be a neural protein (Malatynska et al., 2006). The γ-synuclein has also been found to be over-

expressed in many malignancies and considered a biomarker. It is related to neurodegenerative disorders including, Parkinson's disease, Alzheimer's disease, Amyotrophic lateral sclerosis, Lewy body disease, and vascular dementia (Mukaetova-Ladinska and Lincoln et al., 1999). In addition, it has been reported recently highly expressing in Retinal Ganglion Cells (RGCs) (Surgucheya et al., 2008).

Presence of γ-synuclein in Nervous System

The γ-synuclein was initially found in nervous system tissue and is therefore considered to be a neuronal protein (Zhao et al., 2006). The mRNA levels of synuclein-α, β, and γ were quantified in different brain regions at different ages of mice. The hippocampus, striatum, cortex, and cerebellum were the regions used. The rank order of mRNA for γ-synuclein was hippocampus=striatum>cortex=cerebellum. The rank order was different for both synuclein α and β. The levels varied in each region depending on mouse age. For example, γ-synuclein levels increased significantly only in the cerebral cortex from 5 d to 1 mo of age, but levels in the cerebellum were very high at 5 d and significantly reduced at 1 mo. The different patterns and dynamics of mRNA levels of this family of proteins as the mice aged suggests that they may play a role in development and aging (Malatynska et al., 2006).

In addition, α and γ synuclein proteins have also been found elevated in cerebral spinal fluid (CSF) in elderly individuals with Alzheimer's disease, Lewy body disease, and vascular dementia when compared to normal controls. γ-synuclein had greater elevation

in CSF of patients with Lewy body disease. Elevated synuclein –α and γ were found in CSF of patients in Braak stage III and onward and then remained stable until Braak stage IV (Mukaetova-Ladinska et al., 2008). Braak stages represent a system used to classify Alzheimer patients' stage in the disease. They span from stage I-VI and represent the level of impairment in the patient. Stage I representing no impairment and stage VI representing severe decline (http://www.alz.org/alzheimers_disease_stages_of_alzheimers.asp). Since γ-synuclein is expressed in nervous tissue and found to be highly expressed in nervous tissue of patients with neurodegenerative disease, its DNA sequence was screened to search for possible polymorphism mutations linked with neurodegenerative diseases states, such as Parkinson's disease and ALS. Polymorphisms have been reported but so far none of them is found to be directly correlated with the disease state (Lincoln et al., 1999).

Presence of γ-synuclein in Cancers

Up to now, γ-synuclein has been found in many different types of cancers, including pancreatic adenocarcinoma, uterine papillary serous carcinoma, breast, colorectal, ovarian, lung, liver, gastric cancer, and bladder cancer (Li et al., 2004, Huh et al., 2009, Hibi et al., 2009, Morgan et al., 2009, Liu et al., 2007, Zhao et al., 2006, Yanagawa et al., 2004 and Dokun et al., 2008). Its expression level is correlated to a number of facts including cancer stage, lymph node involvement, metastasis, and perineural invasion.

For example, in a study using 93 breast carcinoma samples, γ-synuclein expression was seen in 36% of all the samples, 81% of the samples from stage III/IV, and 15% of the samples in stage I/II. Cancer stages depend on five factors, which include location of the primary tumor, tumor size and number of tumors, lymph node involvement, cell type and tumor grade, and the presence or absence of metastasis (http://www.cancer.gov/cancertopics/factsheet/Detection/staging). No γ-synuclein expression was seen in normal breast tissue. It was also found that γ-synuclein presence in the primary tumor signified lymph node involvement and metastasis. Again, the presence of γ-synuclein in breast cancer patients had a significantly shorter disease free survival and increased probability of death (Wu et al., 2007).

The trend of γ-synuclein expression and advanced stage cancer is also found in liver and gastric cancer as well. In liver cancer samples γ-synuclein was expressed in 67% of all samples, 5.3% of stage I samples, 88% in stage III/IV samples, and 100% on metastasis samples (Zhao et al., 2006). In gastric cancer samples γ-synuclein was expressed 40% in primary cancers, 51% in primary cancers with lymph node metastasis, 26% in gastric cancer without lymph node involvement, 48% in stage II-IV samples, and 27% in stage I. γ-synuclein was not expressed at all in benign tissue. These also suggest that there is a higher γ-synuclein expression in advanced cancer stages and metastasis phase (Yanagawa et al., 2004).

Using a pancreatic cell line, proteomic and transcriptomic analyses confirmed that γ-synuclein is the only up-regulated protein found in perineural invasion, which is the growth of tumor along nerve branching, providing a path for tumor invasion into new tissue. The expression of γ-synuclein was found correlated to perineural invasion and lymph node metastasis, diminished overall survival, and the strongest negative indicator of disease free survival. Knockdown γ-synuclein expression significantly decreased perineural invasion and liver/lymph nodes metastasis (Hibi et al., 2009).

In conclusion, γ-synuclein is highly expressed in the advanced cancer stages and not expressed at all in normal tissue, except neuronal cells. Therefore, γ-synuclein may be used as a biological biomarker for these cancers.

Regulation of γ-synuclein expression by DNA Methylation

When analyzing the promoter region of γ-synuclein in cancer cell lines where it is overexpressed, this region was found to be hypomethylated at CpG islands. Exogenous overexpression of DNMT3B, a DNA methyl transferase, causes suppression of γ-synuclein mRNA and protein expression in a lung cancer cell line. This caused induction of de novo methylation of γ-synuclein CpG island. When siRNA was used to knock-down DNMT3B there was an increase in γ-synuclein expression. Carcinogens, such as cigarette smoke extract (CSE), have been found to induce down regulation of DNMT3B and lead to γ-synuclein increased expression (Liu et al., 2007).

Similar results were also found in breast and ovarian cell lines, as well as using samples from the patients' bladder, gastric, and liver samples (Gupta et al., 2003, Zhao et al., 2006, Yanagawa et al., 2004 and Dokun et al., 2008). These data have given some insight on how to tackle the problems that γ-synuclein's expression causes. Since a proposed cancer treatment was the use of 5-aza-2'deoxycytidine to prevent the methylation of tumor suppressor genes, it's now found to activate prometastatic genes, γ-synuclein (Ateeq et al., 2008). In conclusion, γ-synuclein found in cell lines in which it is overexpressed is correlated to a hypomethylated promoter region.

Rationale

From above, both PCBP1 and γ-synuclein have been reported to play various roles in the cell. Up to now there is no report in the literatures showing the interaction of these two proteins. Therefore, this study is to examine their physical interaction *in vivo* using mammalian system, and explore the possible functional role of this interaction.

Materials and Methods

Cell Culture

T47D, H292, Mcf-7, HCT116 and HT29 cell lines were obtained from ATCC. NMB (Ko et al., 2005), T47D, and H292 cell lines were maintained in Roswell Park Memorial Institute Medium (RPMI). Mcf-7 cell line was maintained in Dulbecco's Modified Medium (DMEM). HCT116 and HT29 cell lines were maintained in McCoy's media. All medium were supplemented with 10% Fetal Calf Serum and all cell lines were maintained at 37°C in a 5% CO_2 incubator.

Lowry Assay

Using bovine serum albumin as a standard, protein concentrations were determined using Lowry assay. Lysate samples were incubated at room temperature with solution C (1.8mM Na_2CO_3, 98mM NaOH, 0.95mM sodium potassium tartarate, and 0.4mM $CuSO_4$ $5H_2O$) for 10 minutes and further incubated with 1N Folin Ciocalteu's Phenol reagent for 30 minutes. The samples were examined at 660nm using colorimetry to determine the protein concentration.

Western Blot Analysis

Samples were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred to phenylethylsulfonyl fluoride (PVFD) membranes. After a series of washes in .1% and .3% TTBS (Tween-20 Tris

Buffered Saline), the membrane was probed with a specific antibody. The signals were detected using an enhanced chemiluminesence detection system (Amersham Biosciences). The anti-PCBP1 and γ-synuclein antibodies were purchased from Santa Cruz Biotechnology.

Co-Immunoprecipitation

Cell lysate was first incubated with anti-PCBP1 antibody or negative control and then added Protein G-agarose beads. The beads were washed with PBS and then the immunocomplex was eluted using treatment buffer containing SDS.

RNA Isolation

RNA from cells were extracted using Tri-Reagent first and then with chloroform. The mixture was subjected to centrifugation. The top phase containing total RNA was removed. RNA was precipitated using isopropanol.

RT-PCR

First strand cDNA was synthesized using total RNA and the random primer, in the presence of reverse transcriptase. Amplification was performed as 50 minutes at 37°C and 15 minutes at 70°C. PCR amplification of cDNA was performed as 1 minute at 95°C, 35 seconds at 68°C, and 35 seconds at 72°C for 32 cycles. The γ-synuclein primers were as follows: upper, 5'GTGGAGGAGGCGGAGAACATC :lower, 3'CCTCTAGTCTCCCCCACTCTG.

pCR2.1 Cloning

PCR product was cloned into with the cloning site of pCR2.1 vector, purchased from Invitrogen . *E. coli* competent cells were then transformed with the ligation mixture using the heat shocking method. The transformants were then selected using ampicillin LB agar plates.

Small Scale Plasmid Purification

Plasmids were extracted from cultures using Qiaprep Miniprep Kit from Qiagen. Cultures were centrifuged and pellets were resuspended in P1 (50mM glucose, 10mM EDTA, pH 8.5, 25mM Tris HCl, pH 8). They were lysed using Lysis Buffer P2 (0.2M NaOH, 1% SDS), and then neutralized by adding Neutralizing Buffer N3 (5M potassium acetate, glacial acetic acid). The plasmids are separated by centrifugation and applied to Qiaprep spin column, which was washed with Buffer PB and PE. The plasmids were eluted by Buffer EB (10mM Tris HCl, 1mM EDTA, pH 8.4).

Large Scale Plasmid Purification

Bacteria cultures were grown overnight at 37°C. The plasmids were extracted using Qiagen Plasmid Maxiprep Kit from Qiagen. Bacteria cultures were centrifuged and the pellet was resuspended in 10mL of Buffer P1 (50mM glucose, 10mM EDTA, pH 8.5, 25mM Tris HCl, pH8). The bacteria suspension was then lysed by adding 10 mL of Lysis Buffer P2 (0.2M NaOH, 1%SDS), which were further neutralized by adding 10 mL of Neutralizing Buffer P3 (5M potassium acetate, glacial acetic acid). The mixture was

centrifuged at 15,000 rpm. The supernatant was loaded to a Qiagen-tip column, which was then washed with Buffer QC. The plasmid was eluted using Buffer QF, and it was precipitated using isopropanol precipitation method.

Transfection and Reporter Gene Assay

NMB cells were transfected using lipofection method with various concentrations of plasmids containing pcDNA3-γ-synuclein, pcDNA3-SNP γ-synuclein, or blank vector (pcDNA3), individually. The pGL3 plasmid containing the luciferase reporter gene driven by the human MOR promoter was also added in each sample. The pCH110 plasmid was included as an internal standard. Twenty four hours after transfection, cells are harvested and lysed. The luciferase activity was then measured using luciferase reagent (Promega) with a luminometer (Berthold).

Results

Verification of different cell lines expressing γ-synuclein and PCBP1 endogenously

The interaction between PCBP1 and γ-synuclein has been examined via *in vivo* validation using the two-hybrid system as well as using the GST pull-down assay (lab unpublished data). To further determine if this interaction also occurred in the mammalian system, the human cell model system was selected. The literatures were searched first. Based on published data, a total of six cell lines were chosen, including neuroblastoma (NMB) (lab data), lung cancer (H292) (Liu H et al., 2007), breast cancer (T47-D & Mcf-7) (Liu C et al., 2008 and lab data), and colorectal cancer (HCT-116 & HT29) (Liu C et al., 2008), which were reported to express γ-synuclein endogenously. Therefore, the endogenous expression of both PCBP1 and γ-synuclein was examined first using Western blot analysis with cell lysates from these cells and anti-PCBP and anti-γ-synuclein antibodies. Although the monomer form of γ-synuclein is 13kDa, it is reported to have a high tendency to form aggregates (Sung et al., 2007). As in Fig. 1, Western blot analysis showed the aggregated forms of γ-synuclein observed in the different cell lines. In Figure 2 results showed PCBP1 expression in all mammalian cell lines.

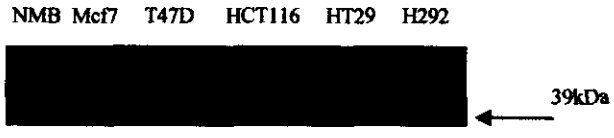

Figure 1: Examination of γ-synuclein expression using six cell lines.
Endogenous expression of γ-synuclein was determined via Western Blot analysis, using T47D, H292, HT29, HCT116, NMB and Mcf-7 cell lysate and anti-γ-synuclein antibody. Arrows indicate the positions of protein markers.

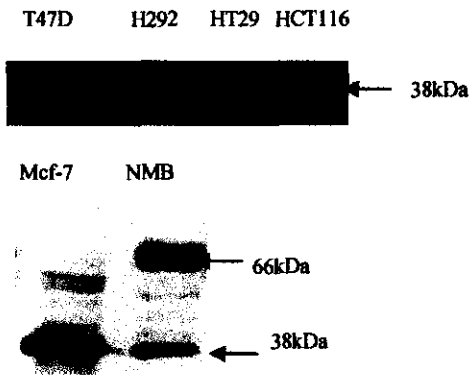

Figure 2: Examination of PCBP1 expression using six cell lines.
Endogenous expression of PCBP1 was determined via Western Blot analysis, using T47D, H292, HT29, HCT116, Mcf-7, and MB cell lysate and anti-PCBP antibody. Arrows indicate the positions of protein markers.

Determination of physical interaction using mammalian cell lines

To examine the PCBP1 and γ-synuclein interaction in the mammalian cell system, co-immunoprecipitation was performed using a PCBP1 specific antibody. This technique can be used to determine the protein-protein interaction, for if an endogenous protein-protein interaction exists, then the PCBP1 antibody will pull down PCBP1 and its interacting proteins. The immunoprecipitated complex will then be able to be examined using SDS-PAGE and Western Blot analysis.

Cell lysate was incubated with anti-PCBP1antibody and without antibody separately as a negative control, and then protein G agarose beads were added. The co-immunoprecipitants were subjected to SDS-PAGE and then Western Blot analysis. In figure 3A, NMB cell lysate was used, and in figure 3B, H292 cell lysate was used. The blots were probed with anti-synuclien antibody and re-probed with anti-PCBP1 antibody to confirm the pull-down of PCBP protein. A γ-synuclein band was seen showing it was

pulled down (shown in Figures 3A and 3B), and also a PCBP1 band was seen showing pull down occurred (data not shown). No bands were seen for cell lysates incubated with no antibody. These results show that when PCBP1 is pulled down, γ-synuclein is pulled down as well, suggesting that a physical interaction between PCBP1 and γ-synuclein is taking place endogenously in these mammalian cell line. (Figure 3) Also, the negative control results validate the co-immunoprecipitation results by showing that without antibody no pull-down occurs.

This procedure was performed using 6 different cell lines, Mcf-7 and T47-D (breast cancer cell lines), HT29 and HCT116 (colorectal cancer cell lines), H292 (lung cancer cell line) (Fig 3B), and NMB (neuroblastoma) (Fig 3A). The same pattern of protein-protein interaction was observed in all cell lines.

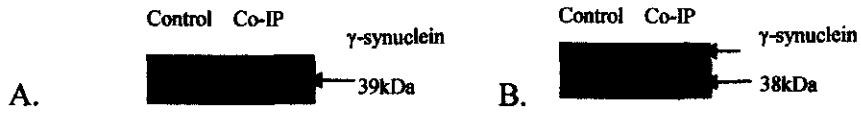

Figure 3: Co-Immunoprecipitation using mammalian cell lines
Figure A shows results using NMB cell lysate and Figure B shows results using H292 cell lysate. The Western blot analysis was performed using γ-synuclein antibody or without antibody. The band of γ-synuclein appeared in both figures, showing that the PCBP1 antibody pulled down γ-synuclein with the PCBP1. Lane 1 is the negative control. Lane 2 is the Co-IP using PCBP1 antibody.

Examining the cDNA sequence of the γ-synuclein in mammalian cell lines

To further confirm the existence of γ-synuclein in these cell lines, the DNA sequences of γ-synuclein were examined using RT-PCR by using γ-synuclein specific primers. The

RNA was isolated from NMB, H292, HT29, HCT116, Mcf-7, and T47D cell lines. RT-PCR was performed and the sizes of PCR products verified using electrophoretic analysis (Figure 4). RT-PCR products were then cloned into pCR2.1 vector. Positive clones containing the PCR products were screened by restriction enzyme digests followed by electrophoretic analysis (Figure 5). The plasmids containing the PCR products were then subjected for DNA sequencing. The DNA sequencing results showed a consistent missense mutation at the C-terminal, when compared with GenBank's published cDNA sequence. The mutation was a T/A mutation causing a valine residue to become a glutamic acid residue

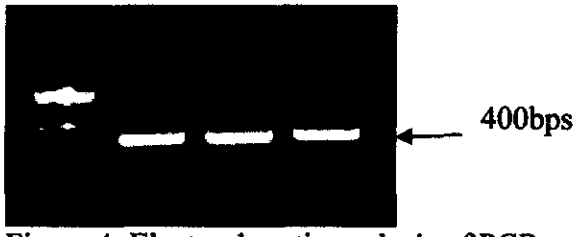

Figure 4: Electrophoretic analysis of PCR products from HCT 116 cell
RNA isolation from cell lysate was performed followed by RT-PCR to amplify the cDNA sequence by using specific primers for γ-synuclein. Agarose gel electrophoresis of the PCR product was performed to verify. A band is seen at approximately 400bps corresponding to the 386bp γ-synuclein cDNA sequence. Lane 1 is the 100bp ladder. Lanes 2-4 represent HCT116 cell lines endogenous cDNA amplified using RT-PCR.

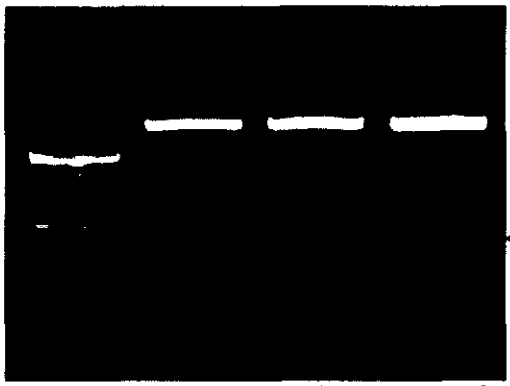

Figure 5: Electrophorectic analysis of RT-PCR cloning in HCT116 cell line

RT-PCR products were ligated with pCR2.1 vector, transformed, and screened. To ensure positive clones contained the γ-synuclein insert, EcoRI digests followed by agarose gel electrophoresis was performed. An insert band is indicated by arrow and a band is seen at a much higher size representing the cut pCR2.1 vector. Lane 1 is the 100bp ladder. Lanes 2-4 represents HCT 116 cell line endogenous cDNA cloned into pCR2.1 vector.

SNP of γ-synuclein

Multiple SNPs (single nucleotide polymorphisms) are available on NCBI's SNP database. A database search was then performed for γ-synuclein and a total of 53 SNPs were found (Table 1), including the missense mutation found expressed endogenously in the mammalian cell lines. Below is a table listing the reference number and sequence of all the SNPs found in the database for γ-synuclein with the SNP matching the missense mutation highlighted.

1	rs71473290	TTCCACAGCCCCTACAGGGACTGTGT[A/G]CAGGGCTAACCCTGAACCTGAGTGG
2	rs74150419	GGAGCTGGGCTCCTGGGGTGCACCAT[G/T]GGGGGTTCCTTGGTAGGGACCCCAT
3	rs74150418	TCGAAGGCCCTGAGCTGCATGCCAAG[A/C]CTCCCTGGCCTCAGAGTCCCTATGT
4	rs74150417	GATGTCTTCAAGAAGGGCTTCTCCAT[A/C]GCCAAGGAGGGCGTGGTGGGTGCGG
5	rs74150416	ACAGGCCTATGTGGCCCTGACCCCTA[C/T]CTAGGAAGCTGGGGACAATGGCCAA
6	rs73353640	AGCCCCGGGTATTGTCTGAATTGCAC[A/G]CCCCCAATACCTCCAGCCCCTCCAA
7	rs73353639	CTGCCCTGCCCCTGGATAGCCTGGCC[A/G]ACTCCCGCCAGCACAGCACTGCCCT
8	rs73353636	GCTCTGCTTTACGCATTGTACGCTTA[C/T]TAGTTCATTTAATAGACACAAAATC
9	rs73353632	ACTACCCTGGCTGGCCCCACAGGGGC[C/T]GCCAACCACACAAGCCAGTTCCTGT
10	rs73334931	AGACAACTTTTCTCAGCCCACTCAGG[A/G]TGGCCCATTAAGGCTGGGGCCTGGA
11	rs73334929	GAATTGCACGCCCCCAATACCTCCAG[C/T]CCCTCCAAGTACAACAAGGCCACGA
12	rs72122064	ACACAGAGGTGACAGTATCCCCGCCT[-/T]CCCCCCCGTCCCCCGCCAGAGGCAG
13	rs71473291	GGCACAGAAGACAACTTTTCTCAGCC[A/C]ACTCAGGGTGGCCCATTAAGGCTGG
14	rs71473289	AGGTGACCATCATCATCATCATCATC[A/C]TGTGTCCCAGGCAGAACACATAGAG

15	rs62621086	CCTGGGGTGCACCATGGGGGGTTCCT[G/T]GGTAGGGACCCCATCCCCCACAGAC
16	rs61856987	GGGCTTCCCAGAGACTGGGGACCAGC[A/T]TGAGGGGTGAAGCTGGGCTCCAGGG
17	rs7090120	CAGCACAGGGTCTCCATGTCCTTGGA[A/G]CTGGCAGCCGCTCCCTCTCCTAGTT
18	**rs9864**	**GCCATCTGCCCCCCAACAGGAGGGTG[A/T]GGCATCCAAAGAGAAAGAGGAAGTG**
19	rs9420407	TCCCTCTCCTAGTTCCCCTTTCTCGT[C/T]GTATGACCTTGAGAAGCAAAGGAAC
20	rs7091084	CTGGCAGCCGCTCCCTCTCCTAGTTC[C/T]CCTTTCTCGTCGTATGACCTTGAGA
21	rs10887683	TCATCAGCAGGATGGGCCCTGGCTCT[A/G]TACACATAGAATGTTGGAAAGCTAT
22	rs3793900	GCTGGCTACCTGTCTGTGCACGCACA[A/C]ACACATTCCCAAGCATACCAGCCTC
23	rs2131395	GGTGGGAGGCGCCGCAGGGTCCACCT[C/T]CTGTCTCTCCTGTGGGATGCCCTTC
24	rs45518038	GAAGCTGGGCTCCAGGGTACTTGGTG[C/T]CCCTGGAAGGCCTCGGCATCATCAG
25	rs35518112	CCCTAGTGGTAAGAAAGCAGTGCTGC[G/T]TCTGACCCTACCCTCTGCAGCTGCC
26	rs35366534	AACACAGAGGTGACAGTATCCCCGCC[-/T]TCCCCCCCGTCCCCCGCCAGAGGCA
27	rs34791897	TGCCCCATGCTGGGTGATTGATGGTA[-/G]GGAGCTGTGCCTCCCTGCCAGCCCC
28	rs34681990	CCTGCATTCGGGTCTGGAATCACCAC[-/A]AACCACCCCTTCGGGGGACTTGGGA
29	rs34020944	GTTGGGAGGTGGCCAGCCCCCTCCAG[A/C]CCATGGAGGGCACGGGGCAGGAGAG
30	rs33979898	CAGGGGACCTGCTGCCCATCCACACT[-/G]CTGGCCAGGAAATGGGGGGCAATTC
31	rs3793899	GCCCTGTGCACACCTATGTGTGTTTG[G/T]CTTTGGCCTCCTCGGGGCCTCTGGG
32	rs1800373	ACATCCATGGTGGGTGTGCAGGGTTG[G/T]GCTGCTGCAGGCAGGACGGAGCTGG
33	rs760112	AAGCCCTACAGACCCCTGCAGACCAT[A/G]AGGCTAAACTAGGGTGGGCGTCTCC
34	rs12355388	CATGCGGCTGCCCACGCTCCTGCCCT[C/T]GTCTCCCTGGCCACCCTTGGCCTGT
35	rs12220121	GAACTGTGAAAGGCCTCAGCAGGGAC[C/T]ATCTGACCACTCATACCCCTGGCCT
36	rs11813415	GGGCCACAGGAAGTGGTCAGGTCCAT[C/G]TCAGGGGACCTGCTGCCCATCCACA
37	rs11550197	AGGGGGTCATGTATGTGGGAGCCAAG[A/C]CCAAGGAGAATGTTGTACAGAGCGT
38	rs11550195	GAGGCGGAGAACATCGCGGTCACCTC[A/C]GGGGTGGTGCGCAAGGAGGACTTGA
39	rs11550194	CCCTCGTCTCCCTGGCCACCCTTGGC[A/C]TGTCCACCTGTGCTGCTGCACCAAC
40	rs11550193	CAGCGTGGATGACCTGAAGAGCGCTC[C/G]TCTGCCTTGGACACCATCCCCTCCT
41	rs11550192	CAAGGAGGGGGTCATGTATGTGGGAG[C/T]CAAGACCAAGGAGAATGTTGTACAG
42	rs7900271	TTCAGCACTGGGCGGCTCAAAGAAGG[C/G]AAGGGACTATTCTGGGGTCACACAG
43	rs7096355	TGCTGTGAATTTTTTTTTTAAATGAT[G/T]CCAAATAAAACTTGAGCCCACTCCT
44	rs7074886	CAGGCCAGCGTGGATGACCTGAAGAG[C/T]GCTCCTCTGCCTTGGACACCATCCC
45	rs7074229	TGCTTCCTCTGGGTGCTGTGTAGCTT[C/G]AGGAGGGGTCCCCAGGTCCTCCCAG
46	rs4520507	GACTCAGATCCTACTCCATGACCTCT[G/T]CCAAGGTCCAGGCCCTCTTACCGTC
47	rs4244973	CCAAGGTCCAGGCCCTCTTACCGTCC[A/T]ACGGGGTCCTTGCCGGTGTCCTCAG
48	rs3750823	CTCTTACCGTCCTACGGGGTCCTTGC[C/T]GGTGTCCTCAGCCTCTGCCTTCCAG
49	rs10232	GGGTGCCCACGCTCCTGCCCTCGTCT[C/T]CCTGGACACCCTTGGCCTGTCCACC
50	rs760113	GAGAAGACCAAGGAGCAGGCCAACGC[C/G]GTGAGCGAGGCTGTGGTGAGCAGCG
51	rs2131396	CAGGCGGGGGCTACCTTGAGGCTGAG[A/C]TAAGCTATCCCCGTGGATGGGACTG
52	rs1802015	CCACCCTCTGGTCCTTCTGACCCCAC[A/T]TATGCTGCTGTGAATTTTTTTTTTA
53	rs1800714	CCAGCTCAAGCCCGCAGCTCGCAGG[A/G]AGATCCAGCTCCGTCCTGCCTGCAG

Table 1: SNPs of γ-synuclein

A total of 53 SNPs were found for γ-synuclein in NCBI's SNP database. Line 18 is the mutation matching our sequence analysis.

Exploring the functional role of PCBP1 and γ-synuclein interaction on the MOR promoter activity

Since the above data showed that PCBP1 and γ-synuclein physically interacted endogenously in the mammalian system, a possible functional role of this interaction can

be examined using the reporter gene assay. Since it is known that PCBP1 can regulate the expression of the mu-opioid receptor gene, this technique can therefore be used to examine if γ-synuclein has any effect on MOR promoter activity via its interacting with PCBP1. Co-transfection was performed using .5, 1, and 2ug of pcDNA3-SNP or DB plasmid or blank vector as a control, along with the luciferase reporter plasmid containing the human MOR promoter. pcDNA3-SNP plasmid contains the DNA sequence of γ-synuclein matching the SNP form and the pcDNA3-DB plasmid contains the DNA sequence of γ-synuclein matching the GenBank database form. The reporter activity using pcDNA3 blank vector is defined as 100%. The preliminary test (Figure 6) showed the pcDNA3-SNP plasmid caused a slight expression increase at .5ug and an expression decrease to about 80% of the control when the concentration was increased, and the pcDNA3-DB plasmid caused an expression increase at .5ug and an expression decrease to about 70% of the control when the concentration was increased. This shows that the interaction between PCBP1 and γ-synuclein may affect MOR promoter expression; however, more experiments need to be performed to further investigate.

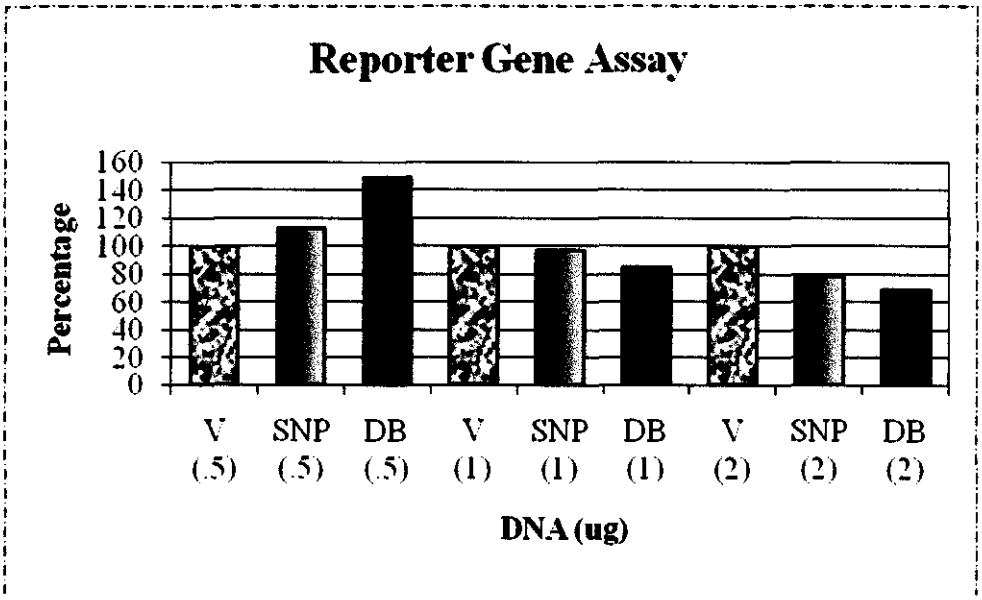

Figure 6: The effects of overexpressing SNP and DB forms of γ-synuclein on the human MOR promoter activities.

The black bars represents data obtained from samples overexpressed with the DB form of γ-synuclein. The light grey bars represent the data obtained from samples overexpressed with the SNP form of γ-synuclein. The black and grey spotted bars represent the data obtained from samples overexpressed with blank vector.

Discussion

Protein-protein interactions affect many things inside the cell, including the change of a protein's function. This concept has been reported for PCBP1, for it binds along with hnRNP K and PCBP2 to c-myc mRNA causing positive translational effect (Evans et al., 2003), while when it binds along with hnRNP A1 and hnRNP K to collagen mRNA it causes mRNA stabilization (Thiele et al., 2004). In this present study one more PCBP interacting protein, γ-synuclein, was discovered by showing that γ-synuclein and PCBP1 physically interact using six different mammalian cell lines found to endogenously express both proteins via a literature search and Western Blot analysis. Co-immunoprecipitation followed by Western Blot analysis demonstrated this interaction. When analyzing the Western Blot data obtained after co-immunoprecipitation, it is seen that PCBP1 only interacts with a form of γ-synuclein approximately trimer in size, approximately 39kDa.

The monomer protein of γ-synuclein is 13kDa in size, but Western Blot analysis of mammalian cell line lysate showed proteins of multiple sizes. There are possible reasons for these results. First, this protein has a high propensity for aggregation (Sung et al., 2007). The higher molecular weight protein expressed may be a multimer of the protein. Second, the other possibility is that the higher molecular weight is due to post translational modifications. Third, γ-synuclein may be also interacting with another protein and form a protein complex. γ-synuclein has been proven to interact with PCBP1 in this study, but it has also been shown to act as a chaperone protein, binding along with

Hsp70 and Hsp90 to ER-α (estrogen receptor α) in the unliganded state in breast cancer. This binding enhances the high affinity ligand-binding capacity of the receptor (Jiang et al., 2004). Since it is know that γ-synuclein interacts with multiple proteins, the higher molecular weight seen may be due to a protein complex. Any of these possible explanations for the higher molecular weight form seen can affect how the protein interacts.

To further validate γ-synuclein in these cell lines, its cDNA was analyzed and a consistent missense mutation was found when compared with GenBank's published cDNA sequence, which coded for a valine residue versus a glutamic acid residue. This missense mutation was found in a SNP database search, with a total of 53 SNPs for γ-synuclein. Since the synuclein family has been found to play a role in neurological disorders, this mutation has been previously investigated using DNA screening and their results indicates that this SNP (glutamic acid residue instead of valine residue) has no direct correlation to the disease in Alzheimer's patients (Luedecking et al., 1999) and no correlation was found for any known polymorphisms for Parkinson's disease or amyotrophic lateral sclerosis (ALS) (Flowers et al., 1999). However, having a non-polar residue versus a polar/acidic residue, may affect the proteins 3D structure as well as possible its binding activity.

To further investigate the role of PCBP1 and γ-synuclein's interaction, the luciferase reporter gene assay was performed to see how their interaction may affect MOR

promoter activity via using both the database and SNP form of γ-synuclein. Preliminary data implicated that the interaction between γ-synuclein and PCBP1 may possibly affect MOR promoter activity, and the database form of γ-synuclein may have a slight stronger effect than the SNP form. However, this experiment needs to be repeated and confirmed.

PCBP1 and γ-synuclein have been shown to interact with each other as well as interact with other proteins separately. It is a possibility that PCBP1 and γ-synuclein's interaction with each other may display different functional role(s) for each of them interacts with the other protein(s). For example, the interaction between PCBP1 and γ-synuclein may affect γ-synuclein's role as a chaperone protein in the binding to the unliganded state of the ERα. Experiments would need to be performed to investigate this, such as co-immunoprecipitation and confocal microscopy to see protein-protein interactions.

In conclusion, this study has identified a physical protein-protein interaction between PCBP1 and γ-synuclein endogenously amongst six mammalian cell lines expressing both proteins. To further investigate this interaction more functional assays, such as the luciferase reporter gene assay need to be repeated. Also, immunofluresence followed by confocal microscopy should be performed to further analyze the interaction as well as the location inside the cell in which it takes place. Using truncated cloned forms of both PCBP1 and γ-synuclein to screen to find the location on each protein in which this interaction takes place should be performed to give more insight on how this interaction works.

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