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Investigation of Poly (C) Binding Protein 1-Interact Proteins by Screening cDNA Library

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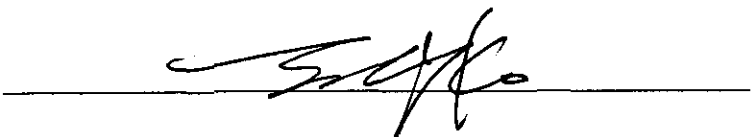

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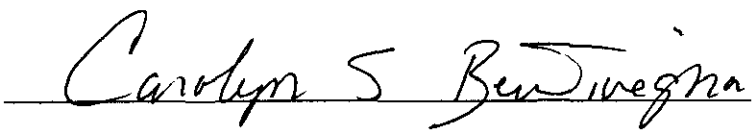

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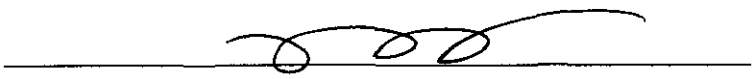

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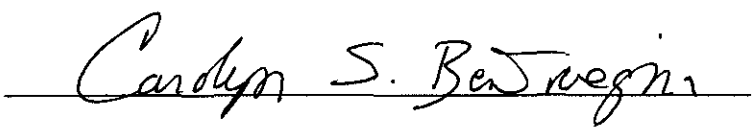

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This thesis not just a representation of research done in a laboratory; it is a depiction of the support and guidance I have received from my mentor, professors, lab colleagues, friends, and family.

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ABSTRACT

Poly (C) binding protein 1 (PCBP1) is a single strand DNA binding protein belonging to the K Homology Domain superfamily. It is a multifunctional protein involved in various aspects of gene regulation including mRNA stabilization, transcriptional and translational control. Recent studies have further demonstrated that PCBP1 participates in the regulation of neuronal μ-opioid receptor gene expression. In order to further understand the multifunctional roles of PCBP1, PCBP1 was used as the bait protein to identify its functional protein partners via protein-protein interactions by screening a cDNA library using the bacteria two-hybrid system.

PCBP1 was cloned into the bait (pBT) vector, which resulted in a bait fusion protein: λcI-PCBP1. The plasmid containing this fusion protein, pBT-PCBP1, was then used to screen over one million clones of a human brain cDNA library on media containing three selective markers (3-AT, chloramphenicol, and tetracycline), and then on a high stringent media containing four selective markers (3-AT, chloramphenicol, tetracycline, and streptomycin). The potential PCBP1 interacting proteins from surviving colonies under high stringent selection were then verified for a protein-protein interaction *in vivo* by extracting and re-co-transforming the target plasmids (containing a cDNA insert from the human brain library) with the pBT-PCBP1 plasmid into bacterial competent cells. Further analysis of the extracted target plasmids using restriction enzyme digestion and gel electrophoresis revealed a 750 base pair cDNA insert. This insert was then cloned into the pGEX vector of the GST Gene Fusion System. The resulting plasmid, pGEX-Clone, was used to over-express the GST-fusion protein in bacterial cells. The over-expression of this protein was confirmed by analyzing bacterial lysates using SDS-PAGE. This protein was then purified through the GST purification system, and now can be used for further investigation of a direct physical interaction with PCBP1.

INTRODUCTION

Poly (C) binding protein 1 (PCBP1) is a single strand (ss) DNA-binding protein that has multifunctional roles in mRNA stabilization (Reimann et al., 2002), transcriptional (Gines-Rivera et al., 2006, Ko and Loh, 2005, and Malik et. al, 2006) and translational (Kim et al., 2005 and Makeyev and Liebhaber et al., 2002) regulation. It belongs to the K Homology domain superfamily, which is made up of two classes of proteins: the hnRNPs and the poly (C) binding proteins (PCBPs), and is involved in many biological processes (Gines-Rivera et al., 2006, Lynch et al., 2005, Malik et al., 2006, Makeyev and Liebhaber et al., 2002, and Moumen et al., 2005)

Heterogeneous Nuclear Ribonucleoproteins (hnRNPs)

In eukaryotic cells, mRNA precursors are packaged with mRNA binding proteins to form heterogeneous nuclear ribonucleoprotein complexes known as heterogeneous nuclear ribonucleoproteins (hnRNPs). hnRNPs are involved in many molecular and cellular functions, including chromatin remodeling, mRNA splicing, transcription, and translation (Bomsztyk et al., 1997, Makeyev and Liebhaber et al., 2002, and Moumen et al., 2005). The hnRNPs also play a role in the cross talk between kinases and factors that mediate nucleic acid directed processes by acting as a docking platform (Inoue et al., 2007). More than 20 hnRNPs have been identified, with hnRNP K being one of the most studied (Bromsztyk et al., 2004, Lee et al., 1996, and Rahman-Roblick et al., 2007).

hnRNP K is found in both the nucleus and cytoplasm of many tissue types (Matunis et al., 1992). It interacts with DNA, RNA, protein kinases, and proteins involved in chromatin remodeling (Denisenko and Bomsztyk, 2002, Dreyfuss et al., 1993, Michelotti et al., 1996, Siomi

et al., 1994, and Tomonaga and Levens., 1996). hnRNP K has multiple domains including three K Homology (KH) domains that mediate binding to DNA/RNA, as well as a highly interactive K protein region (KI) that recruits protein binding partners (Bomsztyk et al., 1997, Bomsztyk et al., 2004, and Paziewska et al., 2004).

The K Homology Domain

KH domains are evolutionarily conserved and bind to short ribonucleotide sequences. The KH domain has a β1-α1-α2-β3-α1 configuration, in which three stranded antiparallel β-sheets are supported by three α-helices (Paziewska et al., 2004). The KH domain was first identified in hnRNP K as an mRNA binding motif. It exists in a variety of RNA-binding proteins either singularly or repetitively, and can function independently or jointly in specific nucleotide binding activities (Malik et al., 2006 and Paziewska et al., 2004). The KH domain family of proteins is able to bind RNA homopolymers such as poly-cysteine (C) residues as well as single stranded (ss) and double stranded (ds) DNA (Leffers et al., 1995, Ko and Loh, 2005, and Malik et al., 2006).

K Homology Domain Superfamily

The KH domain superfamily is made up of two classes of proteins: the hnRNPs and the poly (C) binding proteins (PCBPs). The PCBPs are a family of four proteins PCBP 1-4 (Kim et al., 2005 and Makeyev et al., 2002). Similar to hnRNP K, the PCBPs have three KH domains: two KH domains found at the N-terminus and a third KH domain found near the C-terminus. A third domain found between the second and third KH domain carries the greatest sequence variance. This variable domain contains a nuclear localization signal (NLS) that allows for

shuttling between the cytoplasm and nucleus (Berry et al., 2006). Unlike the PCBPs, the variable domain in hnRNP K has a N-terminal bipartite nuclear localization signal (Chkheidze et al., 1999 and Makeyev and Liebhaber et al., 2002). Both the hnRNP and PCBP family of proteins have multifunctional roles in gene regulation.

Transcriptional Control

The role hnRNP K plays in transcription is well characterized. For example, hnRNP K enhances the expression of *c-myc* by binding to cysteine-rich sequences (the CT element) in the c-myc promoter. hnRNP K also associates with the kappa-B enhancer motif, and enhances expression of the EGR and BRCA1 genes (Stains et al., 2005 and Lynch et al., 2005). In addition, hnRNP K activates and represses RNA polymerase II transcription in a context-dependent manner (Moumen et al., 2005).

PCBPs also play a role in the transcription of specific genes. Recent studies have shown PCBP1 to participate in the transcriptional activation of μ-opioid receptor gene (MOR) by binding to a ssDNA element located in the proximal promoter (Gines-Rivera et al., 2006 and Malik et. al, 2006). Also, PCBP1 was demonstrated to play a regulatory role in *eIF4E* transcription of growth factor-stimulated cells by binding to a region in the promoter containing the eIF4e basal element (4EBE) (Meng et al., 2007).

Posttranscriptional Control

PCBP 1/2 and hnRNP K have been found to mediate mRNA stabilization by binding to pyrimidine-rich motifs (Chkheidze et. al, 1999) such as the cytidine-rich Differentiation Control Element (DICE), a multifunctional *cis*-element (Reimann et al., 2002) found in the 3'

untranslated region (UTR) of many eukaryotic mRNAs. For example, PCBP1 stabilizes Collagen α-1 in hepatic stellate cells (HSC), which are the major cell type responsible for collagen synthesis in cirrhosis of the liver. Collagen α-1(I) mRNA increases during activation of HSCs by a fibrogenic stimulus and stabilization occurs through a RNA complex containing PCBP1 that binds to the C-rich region in its 3' UTR (Stefanovic et al., 1997).

In addition, hnRNP K and PCBP1/2 stabilize reticulocyte-15-lipoxygenase (r15-LOX) mRNA during the differentiation of erythrocytes and granulocytes. A model has been suggested whereby PCBP1 binds to hnRNP K, which binds to DICE of r15-LOX- mRNA 3'UTR. The hnRNP K/PCBP1-DICE complex blocks the 80s ribosome assembly, resulting in translational silencing of r15-Lox mRNA (Ostareck-Ledere and Ostareck, 2004). The same model of translational silencing by PCBP1 has been suggested for human papilomavirus type 16 L2 (L2) mRNA (Makeyev and Liebhaber, 2002).

PCBP1 and 2 have also been shown to stabilize Tyrosine Hydroxylase (TH) and Erythropoietin (EPO) mRNA. A ribonucleoprotein complex binds to the hypoxia-induced protein binding sequence (HIPBS), an RNA stability element in the 3'UTR of TH mRNA. A similar sequence is found in the 3' UTR of EPO mRNA. Both PCBP1 and 2 bind to these sequences, thereby stabilizing the mRNAs for translation (Czyzyk-Krzeska and Bendixen, 1999).

Other mRNAs that are stabilized by the PCBPs include α-globin, poliovirus, and renin mRNA. The stability of α-globin mRNAs has been conferred by binding of an α-complex composed of a single PCBP (either PCBP1 or one of two isoforms of PCBP2) to the pyrimidine rich *cis*-acting stability element in the 3'UTR (Chkheidze et. al, 1999). PCBP 1 and 2 bind and stabilize the 5'-terminal cloverleaf of poliovirus mRNA that serves as a template for viral negative-stranded RNA synthesis for RNA replication (Murry et al., 2001). Renin, also known as

angiotensinogenase, is an enzyme released by juxtanglomerular cells in the kidney in response to low blow volume or decreased NaCl concentration. PCBP1 stabilizes human renin mRNA by binding at the 3' UTR (Morris et al., 2004).

Translational Control

In addition to transcriptional and posttranscriptional controls, PCBPs have been shown to play a role in translational control of folate receptor mRNA and Hepatitus C Virus (HCV) mRNA. PCBP1 interacts with an 18 base cis-element in the 5' UTR of folate receptor mRNA by activating translation in vitro (Anthony et al., 2004). The 5' UTR region of HCV contains a highly structured internal ribosome entry site (IRES) to which PCBPs bind. PCBP 1 and 2 have been shown to interact with IRES in the 5' UTR HCV mRNA (Spangberg, 1999).

Poly Cysteine (C)-Binding Protein 1

As described above, both hnRNP K and the PCBP1 are structurally similar and are involved in transcription and translation of a variety of eukaryotic mRNAs. The exact roles these proteins play are circumstance-dependent. In other words, each protein can have diverse effects on different mRNAs in the same cell or on the same mRNA in response varying environmental signals (Thyagarajan and Szaro, 2004).

PCBP1 has multifunctional roles in mRNA stabilization (Chkheidze et. al, 1999 and Reimann et al., 2002), translational activation and silencing (Anthony et al., 2004 and Spangberg, 1999), and transcriptional regulation (Makeyev and Liebhaber et al., 2002). PCBP1 is a ss DNA-binding protein that was found to participate in μ-opioid receptor gene expression by screening a mouse cDNA library using the yeast one-hybrid system (Ko and Loh, 2005).

PCBP1 recognizes (C)-rich ssDNA through its KH domain (Malik et al, 2006). It is found in both the nucleus and cytoplasm of numerous tissues (Aasheim et al., 1994) including, but not limited to, neuronal tissue (Berry et al., 2006).

Protein-Protein Interactions

The multiple-functionality of the hnRNP and PCBP proteins suggests that their roles may be mediated by protein-protein interactions. Yeast-two-hybrid studies have shown hnRNP K and the PCBPs to interact with different proteins. hnRNP K binds to eIF4E (Lynch et al., 2005) and TATA-binding protein (Michelotti et al., 1996), as well as transcriptional repressors Eed, Zik-1, Kid-1, and MZF-1 (Denisenko et al., 1996, Denisenko and Bromsztyk, 1997, and Bomsztyk et al., 1997). hnRNP K dimerizes and oligomerzies with multiple proteins, including the Src family of tyrosine kinases (Taylor and Shalloway, 1994 and Weng et al., 1994), the proto-oncogene Vav (Bustelo et al., 1995 and Van Seuningen et al., 1995), and with protein kinase C (Schullery et al., 1999). PCBP2 can form homodimers (Gamarnik and Andino, 1997, Kim et al., 2000) and can interact with hnRNP L (Funke et al., 1996, and Kim et al., 2000), hnRNP K and I (Kim et al., 2000), Y-box binding proteins, splicing factor 9G8, and filamin (Funke et al., 1996). In addition, hnRNP K and PCBP2 interact with each other (Kim et al., 2000) and have common protein partners including Y-box-binding protein, splicing factor 9G8, and hnRNP L (Makeyev and Liebhaber, 2002 and Shnyreva et al., 2000).

Several proteins have been shown to interact with PCBP1 in different tissue types, such as hnRNP A2 (Kosturko et al., 20006), Pak1 (Meng et al., 2007), and Lamin A/C (Zhong et al., 2005). Table 1 lists the known protein-protein interactions with PCBP1.

TABLE 1: PCBP1 Interacting Proteins

PCBP1 Interacts with:	Method Used	Reference
c-MYC	TAP/MudPIT	Koch et al., 2007
hnRNP L MATR3 MGC10433 NOVA1 PCBP4 RBM11 SFRS3 PCBP2 THG-1 (TSC22D4) UGP2 CUGBP2 hnRNP K MATR3 QKI RALY SIAHBP1 TNRC4 DDIT4 EWSR1 PDLIM7 PTBP1	Yeast-Two-Hybrid Screen	Lim et al., 2006
hnRNP A2	Yeast-Two-Hybrid Screen In vitro confirmed co-purification experiments	Kosturko et al, 20006
AUF1 (hnRNP D)	Yeast-Two-Hybrid Screen In vitro confirmed co-purification experiments	Kiledjian et al., 1997
PABP-C	Yeast-Two-Hybrid Screen In vitro confirmed co-purification experiments	Wang et al., 1999
P21-activated kinase 1 (Pak1)	Yeast-Two-Hybrid Screen	Meng et al., 2007
Lamins A/C (Progerin)	Yeast-Two-Hybrid Screen	Zhong et al., 2005

The Two-Hybrid System

Protein-protein interactions are vital in cellular processes. The two-hybrid system is an assay that detects these interactions *in vivo.* Other methods, more commonly performed *in vitro,* only allow for the confirmation of the interaction. The two-hybrid system circumvents this disadvantage by allowing for identification of both the protein and its cDNA sequence, which can then be used in further studies. The two-hybrid system can be conducted in bacteria or yeast hosts and is used to detect interacting proteins of a known protein by screening a cDNA library, to confirm a protein-protein interaction between two known proteins, or to identify specific domains of proteins that are involved in protein interactions.

The Yeast Two-Hybrid System

In the first developed yeast two-hybrid system, two different plasmids were used. One plasmid contained the protein of interest fused to the DNA-binding domain of the yeast transcriptional activator protein GAL4. The second plasmid contained the GAL4 activation domain fused to another protein of interest or proteins of a cDNA library. Interaction of these two proteins resulted in the transcriptional activation of a reporter gene containing a binding site for GAL4 (Chien et al., 1991).

The Bacteria Two-Hybrid System

Analogous to the yeast two-hybrid system is the bacteria-two hybrid system. The first developed bacteria-two hybrid system involved the interaction of two fusion proteins, which activated the transcription of the lacZ-reporter gene. The first plasmid encodes a protein composed of the DNA-binding domain (DBD) fused to the known protein of interest. The

second plasmid is composed of library of sequences fused to a subunit of *E. coli* RNA polymerase. A successful protein-protein interaction activates lacZ expression by the DBD binding to the DNA-binding site (DBS) present near the promoter, while the protein of interest must interact with the protein of the library to recruit RNA polymerase to the promoter. The problem with this system is that it uses the *lacZ* as the reporter gene in which candidates are identified by a visual phenotype (i.e. blue color on 5-bromo-4-chloro-3-indolyl-β-D-galactoside plates), and is therefore unsuitable for screening libraries larger than 10^5-10^6 in size (Joung et al., 2000).

An Improved Bacteria Two-Hybrid System

To analyze libraries larger than 10^8 in size, an improved bacteria two-hybrid system was developed that incorporated the Hochschild genetic screen with selectable yeast *HIS3* gene rather than the *lacZ* gene. *HIS3* encodes an enzyme required for histidine biosynthesis that complements a growth defect of *E. coli* cells with a deletion in the homologous *hisB* gene. The compound 3-amino-1,2,4-triazole (3-AT) is a competitive inhibitor of the *HIS3* gene product. Therefore, in the presence of 3-AT, transcription of the reporter gene must be up-regulated to allow bacterial growth. The new system also contains the bacterial *aadA* gene, which confers streptomycin resistance, downstream of the *HIS3* gene, thus acting as a secondary reporter. (Joung et al., 2000).

The bacteria two-hybrid system has been used to successfully identify interacting proteins in both prokaryotic and eukaryotic systems. For example, the 16-kDa subunit c of the vacuolar proton pump was shown to interact with the rat ileal Na^+-dependent bile acid transporter (Asbt) (Sun et al., 2004). Eukaryotic-like serine/threonine kinases, Pkn1 and PknD,

were found to interact with each other in *Chalmydia trachomat* (Verma and Maurelli, 2003). In addition, rshA, and anti-sigma factor, was shown to interact with σ^H, σ^F, and σ^L in *Streptomyces griseus* (Takano et al., 2003).

The goal of this study

Knowing and understanding protein-protein interactions can lead to important discoveries regarding biological pathways and drug discovery. PCBP1 plays a variety of roles in gene regulation. In order to understand the multiple functionality of PCBP1, PCBP1 was used as the bait protein to screen a human brain cDNA library using the bacteria two-hybrid system.

MATERIALS AND METHODS

Bacterial transformation

JM109 competent cells purchased from Invitrogen (Carlsbad, CA) were incubated with plasmid on ice for 30 minutes. Cells were first heat shocked at 42°C and then placed on ice for 2 minutes. LB media was added and cells were incubated at 37°C with shaking at 225 rpm for 60 minutes. Transformants were then plated on LB media containing an appropriate selection marker.

Bacterial co-transformation

XL1-Blue MRF′ Kan strain competent cells of the BacterioMatch® II Two-Hybrid system purchased from Stratagene (La Jolla, CA) was incubated with β-mercapthanol. Each bait plus target vector was then added to the competent cell mixture and placed on ice for 30 minutes. Cells were subject to a heat shock reaction at 42°C and then incubated on ice for 2 minutes. SOC media (tryptone, yeast extract, NaCl, 1 M $MgCl_2$, 1 M $MgSO_4$, 2 M glucose) was added to the cells, which was then incubated at 37°C with shaking at 225 rpm for 90 minutes. Cells were collected and washed with M9+ His-dropout broth (10x M9 salts, M9 media additives (.02% glucose, .02 mM adenine HCl, 10x His dropout amino acid supplement, 1 mM MgSO4, 1 mM Thiamine HCl, .01 mM ZnSO4, 0.1 mM CaCl2, .05 mM IPTG) and then resuspended in M9+ His-dropout broth. Cells were incubated at 37°C for 2 hours and then plated on agar plates containing appropriate selective markers.

Small-scale plasmid purification

Transformants grown overnight under an appropriate selective condition were harvested by centrifugation and plasmids were extracted from transformants using Qiaprep Miniprep Kit from Qiagen (Valencia, CA). Bacterial pellets were resuspended in Resuspension Buffer P1 (50 mM glucose, 10 mM EDTA, pH 8.5, 25 mM Tris HCl, pH 8), followed by addition of Lysis Buffer P2 (0.2 M NaOH, 1% SDS). The pH of the reaction was then neutralized by addition of Neutralizing buffer N3 (5 M potassium acetate, glacial acetic acid). The supernatant was colleted by centrifugation and then applied to a Qiaprep spin column, which was then washed using Buffer PB and PE. Plasmids were eluted by incubation with Buffer EB (10 mM Tris HCl, 1 mM EDTA, pH 8.4) for one minute, and then centrifuged at high speed on a tabletop centrifuge.

Large scale plasmid purification

Transformants inoculated into 250 ml of LB media containing the appropriate selection markers were grown overnight at 37°C with shaking at 225 rpm. The plasmid was extracted from bacterial cells using Qiagen Plasmid Maxiprep kit (Qiagen. Valencia, CA). The cells were harvested by centrifugation at 6000x g and resuspended in Resuspension Buffer P1 (50 mM glucose, 10 mM EDTA, pH 8.5, 25 mM Tris HCl, pH 8). Next, Lysis Buffer P2 (0.2 M NaOH, 1% SDS) was added, followed by Neutralizing Buffer P3 (5 M potassium acetate, glacial acetic acid). The lysates was centrifuged for 30 minutes at $\geq$20,000x g. The supernatant was applied to a Qiagen-tip column, which was then washed with buffer QC. The plasmid was eluted using Buffer QF and precipitated with isopropanol. The DNA pellet was then re-dissolved in water.

Preparation of human brain cDNA library and library screening

A human brain cDNA library in the target plasmid (pTRG) was purchased from Stratagene (La Jolla, CA). Cells containing the library were plated on LB agar containing tetracycline and incubated at 30°C for approximately 24-48 hours. Bacterial cells were collected and pTRG plasmid was extracted using Qiagen Plasmid Maxiprep kit (Valencia, CA).

pBT-PCBP1 was co-transformed with the pTRG containing cDNA inserts from a human brain cDNA library according to bacterial-co-transformation procedure. Transformants were plated on agar plates containing tetracycline, chloramphenicol, and 3-AT, and incubated at 30°C for 48-72 hours. Surviving colonies were then streaked onto another agar plate containing tetracycline, chloramphenicol, and 3-AT, and incubated at 30°C for an additional 24 hours. Colonies that grew on these plates were streaked onto higher stringent agar plates containing tetracycline, chloramphenicol, 3-AT, and streptomycin, and incubated at 30°C for 24 hours. Surviving colonies were then re-streaked onto another high stringent media plate and incubated at 30°C for and additional 24 hours. This procedure was repeated for a total of three streaks on high stringent selection media.

Over-expression and purification of GST-fusion proteins

BL21 competent cells purchased from Invitrogen (Carlsbad, CA) were used for the GST over-expression system. Transformants containing the pGEX plasmid were grown in 2x YTA media (tryptone, yeast extract, NaCl, pH 7) at 37°C for 12-15 hours. Bacteria cultures were diluted 1:100 in fresh 2x YTA media containing ampicillin and grown to approximately A_{600} = 0.8 at 37°C. Protein expression was then induced by addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG). Cells were collected and resuspended in ice-cold 1x PBS (140 mM

NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3). Cells were then incubated with lysozyme at room temperature for 5 minutes and then lysed using 10 cycles of freeze/thaw method. Cell lysates was collected by centrifugation and analyzed using SDS-PAGE.

The GST-fusion protein was purified using Glutathione Sepharose 4B beads purchased from GE Healthcare (Piscataway, NJ) by incubation with cell lysates in the presence of protease inhibitor PMSF at 4°C for 1 hour. Beads were washed with ice-cold 1x PBS and analyzed using SDS-PAGE.

SDS-PAGE and silver staining

Lysates and purified GST-fusion proteins bound to beads were separated on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected with ProteoSilver™ Plus Silver Stain Kit purchased from Sigma (Saint Louis, MO). The SDS-PAGE gel was incubated overnight in fixing solution (ethanol and acetic acid). The gel was then washed with a 30% ethanol solution, followed by a water wash. The gel was incubated with Sensitizer solution, and then was washed twice. The gel was subject to silver equilibration with Silver solution and then developed with Developer solution until bands were apparent. The reaction was stopped using ProteoSilver Stop Solution.

RESULTS

Studies have shown Poly (C) binding protein 1 (PCBP1) to be involved in several aspects of gene regulation. In order to further understand the multifunctional roles of PCBP1, it is important to identify its interacting protein partners. In this study, the bacteria two-hybrid system was used to identify the PCBP1 binding-protein partners.

Cloning of PCBP1 into the (pBT) bait vector

The bacteria two-hybrid system detects protein-protein interactions *in vivo*. When two proteins interact, the transcription of a reporter gene is activated allowing for the identification of positive clones. The cDNA sequence of a protein of interest, termed the bait protein, is cloned into bait (pBT) vector. This 3.2 kb bait plasmid has a low copy p15A replication origin and a gene for chloramphenicol resistance. The plasmid also has a full-length bacterial phage λcI protein that is under the control of IPTG-inducible *lac-UV5* promoter (see Figure 1A).

The bait vector has a multiple cloning site (MCS) to facilitate the cloning of the gene of interest, in this case PCBP1. This vector produces a bait fusion protein: PCBP1 fused to the 237 amino acid bacteriophage λ repressor protein (λcI) containing the amino-terminal DNA-binding domain and the carboxy-terminal dimerization domain. The DNA binding-domain of λcI binds to the λ operator sequence located upstream of the reporter genes *HIS3* and *aadA*.

PCBP1 cDNA sequence was cloned into the bait (pBT) vector. The resulting plasmid, pBT-PCBP1 was transformed into XL1-Blue MRF' competent cells and subject to chloramphenicol selection. pBT-PCBP1 was extracted from surviving colonies and analyzed using restriction enzymes Nco I and Xho I. As shown in figure 1B, three bands of the predicted sizes (2.9 kb, 1.4kb, and 600 bp) were observed using gel electrophoresis with ethidium bromide

staining. The pBT-PCBP1 plasmid was then analyzed by DNA sequencing to confirm the correct in-frame sequence of PCBP1 cDNA with the λcI protein.

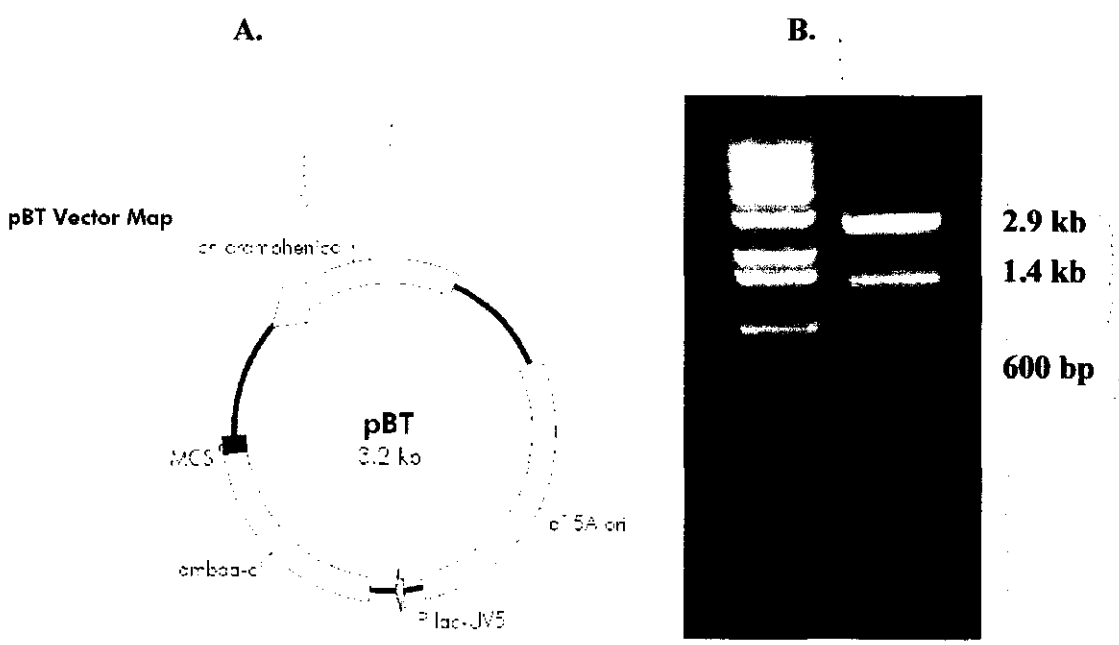

Figure 1: Cloning of PCBP1 into pBT vector.

A. Diagram of 3.2 kb bait (pBT) vector. Vector map (adopted from Stratagene BacterioMatch® II Two-Hybrid System) showing the p15A replication origin, Multiple Cloning Site (MCS), λcI gene, and chloramphenicol-resistance gene. Insertion of PCBP1 cDNA in the MCS resulted in a bait fusion protein: PCBP1 fused to bacteriohage λ repressor protein (λcI) containing the amino-terminal DNA-binding domain and the carboxy-terminal dimerization domain. **B. Confirmation of the presence of PCBP1 cDNA insert in bait (pBT) vector.** The pBT-PCBP1 plasmid was digested with Nco I and Xho I restriction enzymes and subjected to electrophoresis analysis using ethidium bromide staining to confirm the presence of three bands of approximately 2.9 kb, 1.4 kb, and 600 kb in size.

Self-activation test of the λcI-PCBP1 fusion protein

To prevent a high background (i.e. a high number of false positives) during the library screen, the bait fusion protein must be tested to determine if it can self-activate the reporter genes: *HIS3* and *aadA*. The 4.4 kb target (pTRG) vector (Figure 2A) is used in the co-transformation and contains the amino-terminal domain of RNA polymerase α subunit, which is under the control of IPTG-inducible tandem promoter *lpp/lac-UV5,* a low-copy ColE1 replication origin, and a tetracycline-resistance gene.

The bait fusion proteins are tested for self-activation, as some bait proteins are known to activate transcription of the reporter genes with out an interacting partner. Also, there is a chance that the bait protein may be toxic to the host, and could therefore impact the ability of the host to survive on selective media. The bait protein may also strongly or weakly activate the transcription of the reporter genes. If the bait protein strongly activates the transcription of the reporter genes, a high background may result. Weak interactions may result in the lack of transcriptional activation, thereby affecting the results of the library screen.

In theory, the reporter genes should not be activated when the recombinant bait vector is co-transformed with an empty target vector (pTRG-Empty). This is because pTRG-Empty does not contain a target protein that can interact with the bait fusion protein. Thus, bacteria are unable to grow on media containing selective markers for the reporter genes. However, if the recombinant bait plasmid is capable of activating the reporter genes on its own, bacterial growth is observed on media containing the appropriate selective markers (3-AT, chloramphenicol, and tetracycline), and for that reason modifications are need to certain residues or domains.

The recombinant bait plasmid, pBT-PCBP1 was tested for self-activation of the reporter genes by co-transformation with the pTRG-Empty vector (does not contain a fusion protein) into

bacteria competent cells and plated on media containing three selective markers: 3-AT, chrolamphenicol, and tetracycline. As shown in Figure 2B, colonies were not observed confirming that pBT-PCBP1 is a suitable for screening a cDNA library.

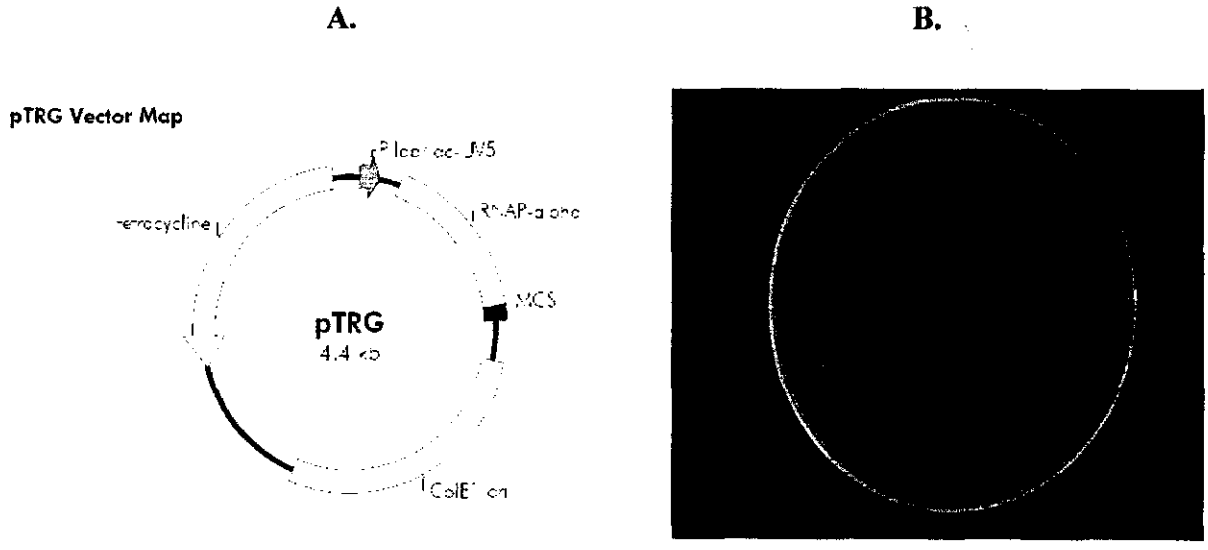

Figure 2: Self-activation test of the recombinant bait plasmid pBT-PCBP1.

A. Diagram of the 4.4 kb target (pTRG) vector. Vector map (adopted from Stratagene BacterioMatch® II Two-Hybrid System) showing a low copy Col1E1 replication origin, tetracycline resistance gene, and the gene for the RNA polymerase-α subunit. Empty vector (pTRG-Empty) does not contain a fusion protein. **B. Self-activation test.** pBT-PCBP1 was co-transformed with pTRG-Empty into competent cells and plated on media containing 3-AT, tetracycline, and chloramphenicol. Colonies were not observed indicating that pBT-PCBP1 does not self activate the reporter gene and can be used to screen a cDNA library.

Setup of bacteria two-hybrid system using positive and negative controls

Before screening the human brain cDNA library, pilot experiments were conducted in order to set up the bacteria two-hybrid system. This was done by co- transforming pBT-LGF2 with pTRG-Gal11^{P} as the positive control, as well as pBT-Empty with pTRG-Empty as the negative control. The bacterial transformations were plated on media containing three selective markers: 3-AT, chloramphenicol, and tetracycline.

The pBT-LGF2 plasmid is a 3.3 kb vector that contains the 40 amino acid dimerization domain of the yeast transcriptional activator Gal4. This vector produces a Gal4-λcI fusion protein. The 4.6 kb pTRG-Gal11^{P} vector encodes a 90 amino acid domain of the Gal11 protein. The dimerization domain of the Gal4 protein has been shown to interact with the Gal11 protein in *E. coli* cells.

Co-transformation of both vectors (see figure 3A) resulted in a protein-protein interaction that activated transcription of the reporter genes *HIS3* and *aadA,* allowing the bacterial cells to survive on media containing 3-AT. As shown in Figure 3B, co-transformation of the positive control plasmids resulted in the growth of a significant number of bacterial colonies.

The negative control was performed using the empty bait (pBT-Empty) (see Figure 1A) and target (pTRG-Empty) vectors (see Figure 2A); these vectors do not contain fusion proteins that interact. Hence, the reporter gene is not transcriptionally activated and bacterial cells are unable to grow in the presence of 3-AT. As shown in Figure 3C, the co-transformation of empty bait and target vectors resulted in a lack of bacterial growth.

The results from positive and negative control experiments indicated the successful set up of the bacteria-two hybrid system.

A.

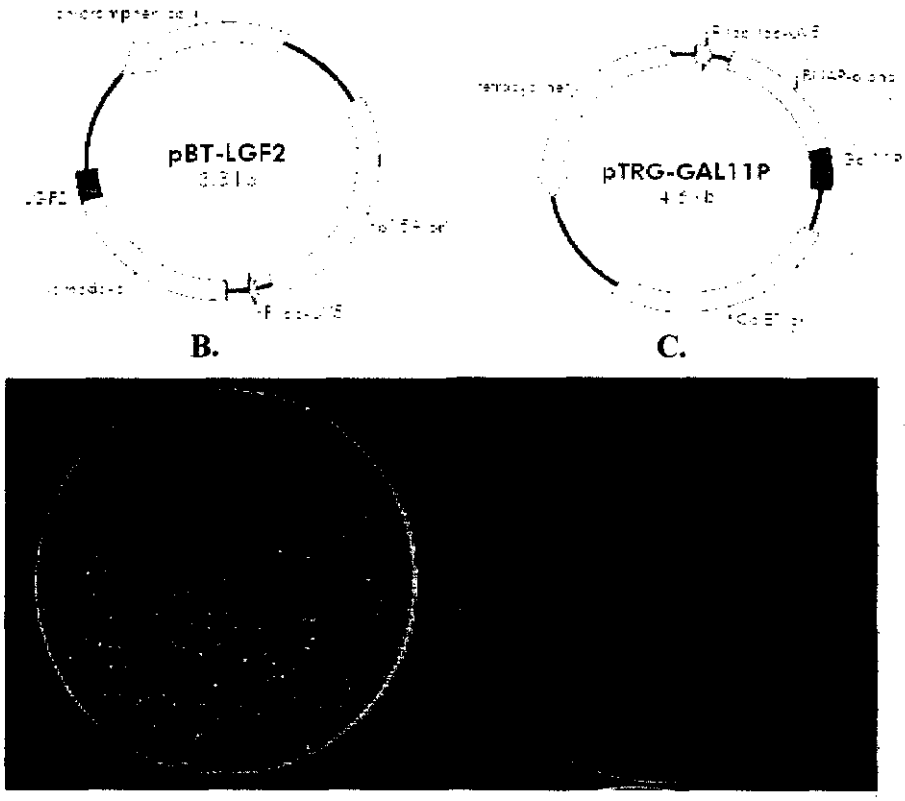

Figure 3: The setup of positive and negative controls using the two-hybrid system.

A. Vector maps of positive control pBT-LGF2 and pTRG-Gal11P plasmids. (Adopted from Stratagene BacterioMatch® II Two-Hybrid System). The pBT-LGF2 contains the dimerization domain of the yeast transcriptional activator GAL4, while the pTRG-Gal11^P contains the Gal11^P protein. Gal4 has been shown to bind to Gal1^P in *E. coli*, thereby activating the *HIS3* and *aadA* reporter genes that allow for the growth of bacterial colonies on media containing 3-AT. The empty bait and target vectors (see figure 1A and 2A) do not contain fusion proteins that are capable of interacting. Therefore, the reporter genes are not activated and bacterial cells cannot grow in the presence of 3-AT. **B. Positive control plate.** pBT-LFG2 was co-transformed with pTRG-Gal11^P into bacterial cells, which were then plated on media containing 3-AT, chloramphenicol, and tetracycline. A Significant number of colonies were observed indicating a successful positive control pilot experiment. **C. Negative control plate.** Bacterial cells co-transformed with pBT-Empty and pTRG-Empty were plated on media containing 3-AT, chloramphenicol, and tetracycline. Colonies were not observed indicating a successful negative control pilot experiment.

Screening of the human brain cDNA library

In order to screen a human brain cDNA library for protein interacting partners of PCBP1, pBT-PCBP1 was co-transformed with target vectors (pTRG-cDNA) containing a cDNA library into bacteria cells. Approximately 1.65 x 10^6 clones were screened. Positive control experiments using pBT-LGF2 and pTRG-Gal11^P vectors, as well as negative control experiments using pBT-PCBP1 with pTRG-Empty were performed simultaneously. As shown in Figure 4, putative positive colonies were observed on media containing three selective markers (3-AT, chloramphenicol, and tetracycline).

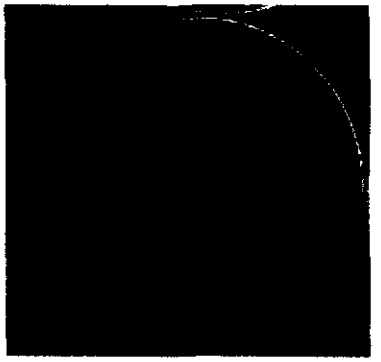

Figure 4: Screening a human brain cDNA library.

An example of a cDNA library screening plate. pBT-PCBP1 was co-transformed with pTRG-cDNA containing human brain cDNA library and plated on media with three selective markers: 3-AT, chloramphenicol, and tetracycline.

Verification of putative positive colonies from library screen

To verify a potential protein-protein interaction, colonies that grew on media containing three selective markers (Figure 4) were streaked onto higher stringent plates containing four selective markers: 3-AT, chloramphenicol, tetracycline, and streptomycin. As shown in Figure 5, only some colonies that were able to grow on plates containing three selective markers were also able to grow under a higher selective condition.

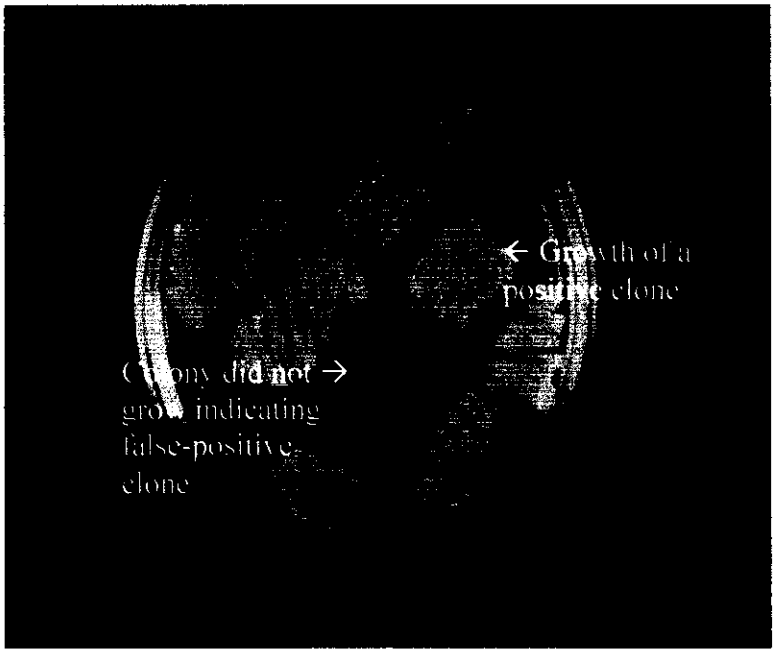

Figure 5: Screening candidate clones using a high stringent screening condition.

Bacterial cells co-transformed with pBT-PCBP1 and pTRG-cDNA plasmids containing cDNAs from a human brain library were plated on media containing 3-AT, chloramphenicol, and tetracycline. The transformants that grew on this on this media were then streaked onto high stringent plates containing four selective markers: 3-AT, chloramphenicol, tetracycline, and streptomycin. Only some of colonies from the library screening were able to grow under high stringent conditions.

***In vivo* validation of protein-protein interaction and analysis of the cDNA insert from target vectors of candidate clones**

In order to validate the protein-protein interaction in individual clones obtained from high stringent plates, the pTRG plasmid containing a cDNA sequence from a library was extracted and co transformed with the recombinant bait plasmid, pBT=PCBP1.

The candidate clones from high stringent selection plates were first cultured in Luria Broth containing tetracycline (LB-Tet). This allowed for the amplification of only the pTRG-cDNA plasmid for the reason that the pBT-PCBP1 vector only confers chloramphenicol resistance. The pTRG-cDNA plasmid was then extracted using DNA plasmid purification methods and then co-transformed with pBT-PCBP1. Co-transformants, according to manufacture's suggested protocol, were plated on media with 3=AT, tetracycline, and chloramphenicol to confirm a protein-protein interaction *in vivo.*

The target plasmids containing cDNA inserts from verified positive colonies were then subjected to restriction enzyme analysis. These plasmids were digested with Xho I and Eco R1 enzymes and analyzed by gel electrophoresis with ethidium bromide staining. As shown in Figure 6, a 750 base pair insert was identified, which was termed pTRG-Clone.

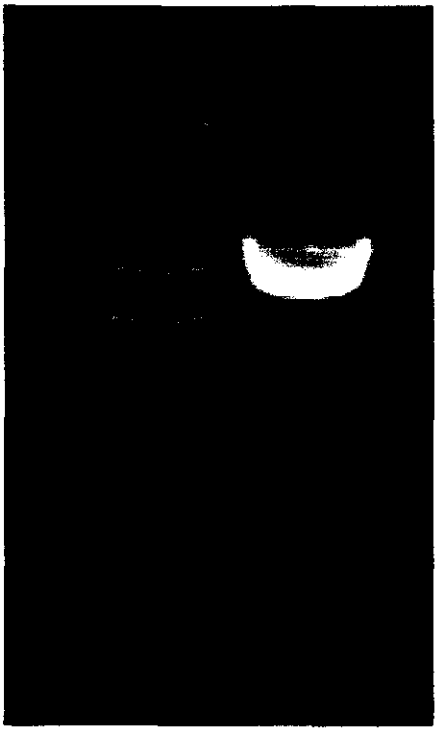

Figure 6: Restriction enzyme analysis of pTRG-Clone.

Target plasmid, extracted and isolated from verified candidate clones was subject to restriction enzyme analysis using Xho I and Eco R1 restriction enzymes. Gel electrophoresis with ethidium bromide staining showed a band of approximately 750 base pairs in size.

Confirming physical interaction using the GST Gene Fusion System

To further confirm the physical protein-protein interaction of the candidate protein (from pTRG-Clone) with PCBP1, the *in vitro* GST Gene Fusion System was used. This system uses the GEX vector to produce Gluthathione-S-Transferase (GST) fusion proteins that can be expressed and purified from bacterial cells.

Cloning of cDNA insert into pGEX plasmid

The pGEX vector is used in the GST protein purification system. pGEX contains the gene for *Schistosoma japonicum* Glutathione S-Transferase (GST) protein and a gene that confers ampicillin resistance (Figure 7A). The pGEX vector also contains the *tac* promtoter, which can be induced by the lactose analog isopropyl-β-D-thiogalactoside (IPTG), and a *lac*Iq gene, whose product binds to the operator region of the *tac* promoter acting as a repressor protein, thus, preventing expression until induced by IPTG.

In order to over-express the candidate protein in bacteria cells, the cDNA sequence from the pTRG-Clone plasmid was cloned into the pGEX vector. This vector produced a fusion protein: GST-candidate protein, which was termed GST-Clone.

The successful cloning of cDNA into the pGEX vector was confirmed using digestive enzyme analysis with Xho I and Eco R1 enzymes. As shown in Figure 7C, gel electrophoresis analysis with ethidium bromide staining confirmed the presence of a 4.9 kb and 750 bp band. As a control, an empty pGEX vector was subject to restriction enzyme analysis using Xho I and one band of approximately 4.9 kb (Figure 7B) was seen.

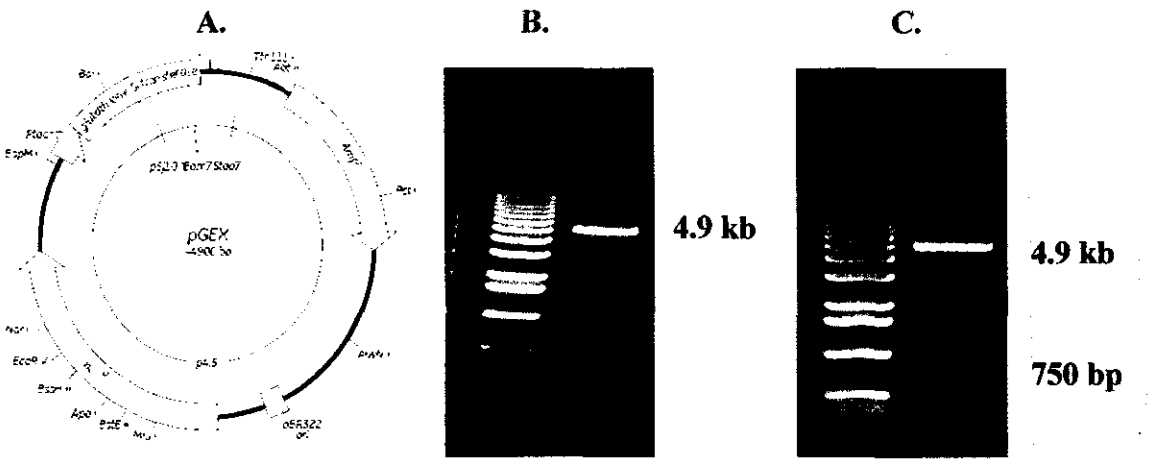

Figure 7: Cloning of cDNA into the pGEX vector (pGEX-Clone).

A. Vector map of the pGEX vector. The pGEX vector contains the gene for *Schistosoma japonicum* GST protein and a gene that confers ampicillin resistance (vector map adopted from GE Healthcare GST Gene Fusion System). Expression of this vector in *E. coli* cells yields a fusion protein with GST in the amino-terminal and the protein of interest in the carboxy-terminal. **B. Restriction enzyme analysis of pGEX vector.** The pGEX vector was subject to restriction enzyme analysis using Xho I and gel electrophoresis with ethidium bromide staining. One band of approximately 4.9 kb was observed. **C. Restriction enzyme analysis of pGEX-Clone plasmid containing the cDNA of interest.** To confirm the presence of the cDNA insert, the pGEX-clone plasmid containing GST-candidate fusion protein (GST-Clone) was subject to restriction enzyme analysis using Xho I and Eco R1 and gel electrophoresis with ethidium bromide staining. Two bands of approximately 4.9 kb and 750 bp were observed indicating that the cDNA was successfully cloned into the pGEX vector.

Over-expression of GST-Clone protein

In order to over-express and purify the GST fusion protein, the pGEX and pGEX-Clone vectors were transformed into *E. coli* BL21 competent cells separately. These cells are protease-deficient cells and are designed to maximize expression of full-length fusion proteins. Cells transformed with pGEX vectors were grown in 2x YTA media containing ampicillin and treated with IPTG. Treatment with IPTG ensures maximal over-expression of the GST fusion proteins.

The bacteria cells were collected and lysed. Lysates were analyzed using SDS-PAGE with silver staining for the presence of the over expressed GST-fusion protein, GST-Clone.

GST is approximately a 29 kDa protein and GST-Clone is approximately a 43 kDa protein. As shown in Figure 8 Lane 1, a dark band at 29 kDa was observed in the lysate from pGEX-transformed cells. Lanes 2 and 3 show a dark band at approximately 43 kDa from bacterial cells transformed with pGEX-Clone. These results indicate the successful over-expression of the GST-Clone protein.

Purification of GST and GST-Clone proteins

After confirmation of the presence of over-expressed GST fusion protein, GST-Clone, the lysates were then subject to GST-purification using Glutathione Sepharose 4B beads. The GST protein acts as a tag that can be isolated using affinity chromatography to purify GST-fusion proteins. Glutathione, which acts as a ligand, is immobilized to a matrix such as Sepharose beads. Bacterial lysates are applied to the matrix, thus allowing GST-fusion proteins to bind to the ligand. The beads are then washed to remove any impurities and unbound protein, resulting in purified GST-fusion protein bound to Sepharose beads. As shown in Figure 8 Lanes 5-7, GST and GST-Clone were successfully purified from bacterial lysates at the expected sizes.

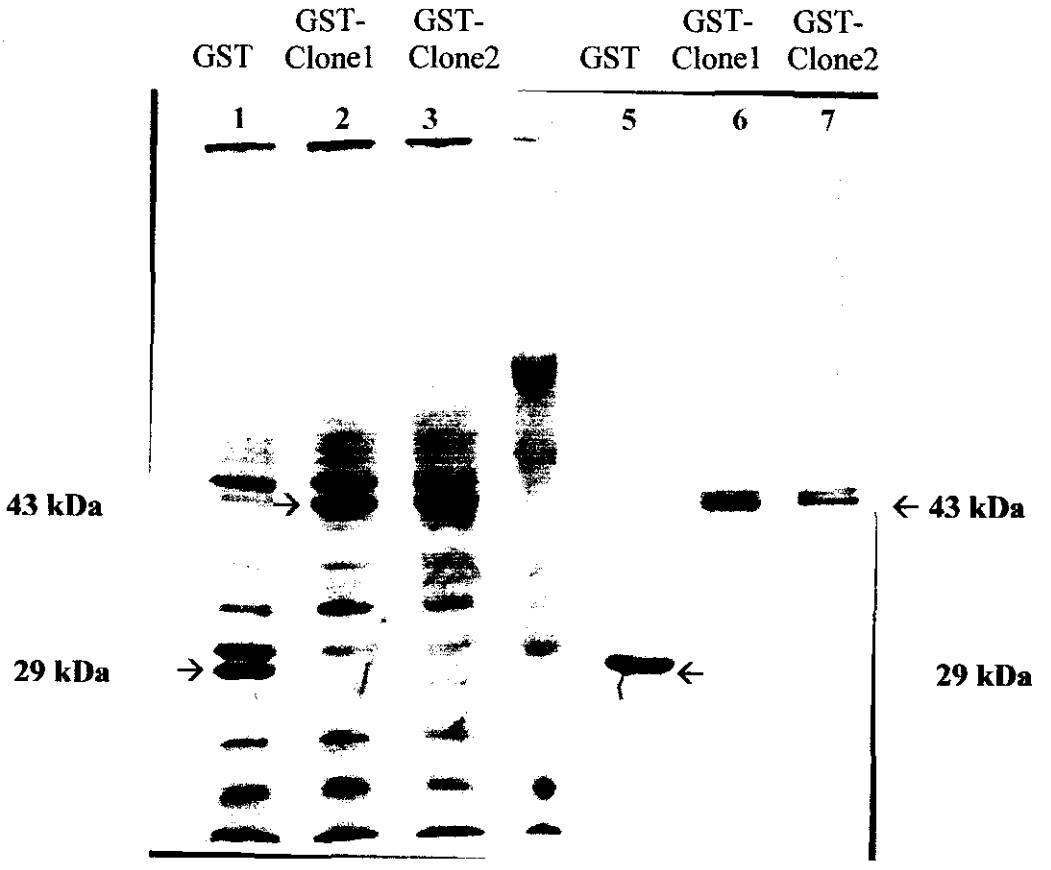

Figure 8: Silver stain analysis of bacterial lysate containing GST and GST-Clone.

Bacterial lysates from cells transformed with pGEX or pGEX-clone were analyzed for the GST and GST-fusion protein: GST-Clone (29 kDa and 43 kDa, respectively) using SDS-PAGE analysis with protein silver staining (Lanes 1-3). The GST and GST-clone proteins were purified from bacterial lysates using Glutathione Sepharose 4B beads. The expected size bands at 29 kDa for GST and 43 kDa for GST-Candidate Clone protein were observed (Lanes 5-7).

DISCUSSION

In order to understand PCBP1's multiple functions, it is important to identify its interacting proteins given that protein-protein interactions are essential to many protein functions in biological processes. One method of identifying protein-protein interactions is the two-hybrid system, which can be conducted in bacteria or yeast hosts. The bacteria two-hybrid system has certain advantages such as the ability to analyze larger libraries as a result of higher transformation efficiencies, faster growth rates (as observed in *Escherichia coli*), and a lower false-positive rate of approximately 3×10^{-8}. False-positives pose a difficult problem in yeast two-hybrid screens due to the chance that the yeast host may harbor a eukaryotic homologue of one of the interacting partners and thereby activate the transcription of the reporter genes. In addition, the bait fusion proteins require a nuclear localization signal that may interfere with target-binding activities (Chien et al., 1991 and Joung et al., 2000).

Therefore, in this study we used the bacteria two-hybrid system to screen for PCBP1-interacting protein partners. The bait fusion protein, λcI-PCBP1, did not self-activate transcription of the reporter genes and was used to screen a human brain cDNA library on media containing three (chloramphenicol, tetracycline, 3-AT) and four (chloramphenicol, tetracycline, 3-AT, streptomycin) selective markers. The pTRG-cDNA plasmids from candidate clones that survived high stringent selection were extracted and verified for an *in vivo* protein-protein interaction by co-transformation with pBT-PCBP1. The pTRG-cDNA plasmids from verified clones were analyzed, revealing a 750 base pair cDNA insert.

Various PCBP1-interacting proteins have been reported in different earlier yeast two-hybrid studies (as listed in Table 1). There are several possibilities for these differences. For example, different genes are expressed in diverse tissues and the genes being expressed in a

given host may also vary due to the host's age, race, sex, and environment. Consequently, the variations observed may be a result of examination of the protein-protein interactions using different tissue types. For example, the identification of Lamins A/C as a PCBP1-interacting protein was detected in lymphoblasts (Zhong et al., 2005), while hnRNP A2 was observed to bind to PCBP1 in oligodendrocytes (Kosturko et al., 2006). Another possible reason may be due in fact that some studies used a different protein as bait, such as *c-MYC* (Koch et al., 2007) and Pak 1 (Meng et al., 2007). Also, the bait plasmid of the yeast-two hybrid kit varied, which may have affected the binding properties of the bait fusion protein as a result of in-frame sequencing with the cDNA sequence of PCBP1. For example, AUF1 was identified as a PCBP1 interacting protein using the pGBD plasmid (Kiledjian et al., 1997) and the pDONR223 vector of the Gateway system was used to identify several other proteins such as hnRNP K (Lim et al., 2006).

Furthermore, variations may also be due to the cDNA library being used, which may not represent all genes due to the construction of the cDNA library from different manufacturers. In addition, the binding properties of target proteins can vary as a result of in-frame sequencing to generate fusion proteins. For example, PCBP1 was detected as a hnRNP A2 binding protein from a Matchmaker™ Pre-transformed human cDNA library obtained from Clonetech/BD (Kosturko et al., 2006), whereas a number of other PCBP1-interacting protein were identified from screening a human brain cDNA library purchased from ProQuest, Invitrogen (Lim et al., 2006). While several PCBP1-interacting proteins have been identified through the yeast two-hybrid system, not all have been verified indicating there are more PCBP1-interacting proteins yet to be discovered.

In this study, a candidate PCBP1-interacting protein has been found and verified for an *in vivo* protein-protein interaction with PCBP1 through a re-co-transformation test of the pBT-

PCBP1 and pTRG-Clone plasmids. This protein-protein interaction should be further analyzed in additional *in vivo* and *in vitro* assays. The reason for this is that there may be factors in the host's environment (i.e. bacteria) that may mediate or enhance the protein-protein interaction that may not be present in other cellular environments. The examination of a direct interaction between the candidate protein and PCBP1 *in vitro,* such as a GST pull down assay, is still necessary. For this reason, the GST-fusion protein, GST-Clone, was established for *in vitro* testing, which may help to identify the interaction without the disturbance of certain environmental influences that may occur *in vivo*. Further *in vivo* experiments will allow for confirmation of a protein-protein interaction in the proteins' actual cellular environment where it is possible that the bait and target proteins may be modified by factors in the cells that can either enhance or impair the interaction. Also, it may be possible to identify proteins (i.e. in the form of a signaling cascade) that mediate or interfere with the interaction, as well as identify different isoforms of the same proteins.

The identification of the candidate protein may help to understand the physiological roles of PCBP1, as well as the role of the candidate protein in cellular processes. In particular, any signaling cascades affecting the protein-protein interaction that may lead to identifying new functions and targets for therapeutic usages.

CONCLUSION

In summary, the bacteria two-hybrid system was used to identify the protein-interacting partners of PCBP1. PCBP1 was first cloned into the bait vector (pBT-PCBP1) and analyzed for in-frame sequence with the λcI protein by DNA sequencing. Results of the co-transformation of pBT-PCBP1 with an empty target vector (pTRG-Empty) verified that the λcI-PCBP1 fusion protein did not self-activate the reporter genes (*HIS3* and *aadA)* and was used to screen a human brain cDNA library. In addition, the bacteria two-hybrid system was successfully setup using pilot experiments with positive (pBT-LGF2 and pTRG-Gal11P) and negative control (pBT-Emtpy and pTRG-Empty) plasmids.

Approximately 1.65 x 10^6 clones of a human brain cDNA library were screened using media containing three selective markers: 3-AT, chloramphenicol, and tetracycline. In order to verify positive clones, colonies that grew on this media were further screened using high stringent media containing four selective markers: 3-AT, chloramphenicol, tetracycline, and streptomycin. The candidate clone containing the cDNA insert (pTRG-Clone) was isolated and extracted. Restriction enzyme analysis demonstrated that the candidate clone contained a 750-base pair cDNA insert. pTRG-Clone was then re-co transformed with pBT-PCBP1 into bacteria competent cells to validate a positive protein interaction *in vivo.*

To further confirm the physical interaction of the candidate protein with PCBP1, the 750 base pair cDNA insert was cloned into the pGEX vector and over-expressed in bacterial cells. Expression and purification of the GST-fusion proteins (GST and GST-Clone) were confirmed by SDS-PAGE analysis with silver staining.

Future experiments are necessary to verify the candidate protein's physical interaction with PCBP1 and to identify the functional roles of this interaction.

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