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Identification of the Interaction Between RACK1-PCBP1 and Characterization of the Novel Functional Effect of the Interaction

Pranjal Nahar-Gohad

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Pranjal Nahar-Gohad

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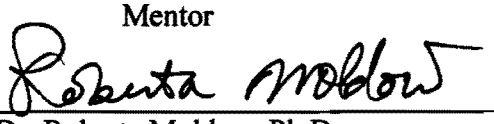
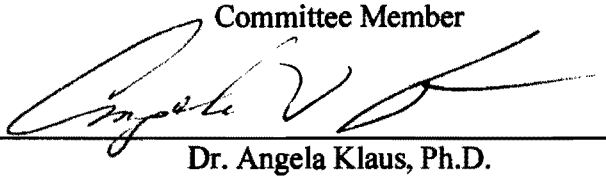
APPROVED BY

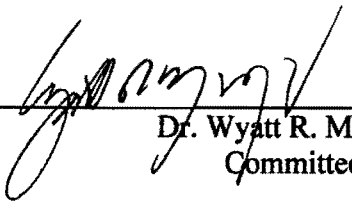
Dr. Jane L. Ko, Ph.D.
Mentor



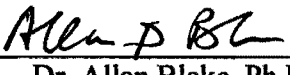
Dr. Roberta Moldow, Ph.D.
Committee Member



Dr. Angela Klaus, Ph.D.
Committee Member



Dr. Wyatt R. Murphy Jr, Ph.D.
Committee Member



Dr. Allan Blake, Ph.D.
Committee Member & Director of Graduate Studies

Dr. Jane L. Ko, Ph.D.
Chair, Department of Biological Sciences

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ABSTRACT

Poly (C) binding protein 1 (PCBP1) is a single stranded DNA binding protein that belongs to the K Homology (KH) domain superfamily of proteins. Studies have shown that PCBP1 is involved in the regulation of μ-opioid receptor and translational regulation of several other proteins. In order to understand the versatile functional roles of PCBP1, PCBP1 interacting proteins were identified by screening a human brain cDNA library using a bacterial two hybrid system. One of the positive clones was sequenced and identified as RACK1 protein. The goal of this study is to characterize the physical interaction between PCBP1 and RACK1, and study the functional effect. The *in vivo* interaction between these two proteins was validated by a bacterial two hybrid system, and the physical interaction in the mammalian system was confirmed using a co-immunoprecipitation assay. RACK1 has seven tryptophan and aspartic acid (WD) domains. Several WD truncated forms of RACK1 were constructed and the binding of PCBP1 with RACK1 constructs was mapped at the WD7 domain. RACK1's functional role was further examined by a luciferase reporter assay, where overexpression of RACK1 decreased the μ-opioid receptor (MOR) promoter activity. In addition, a significant increase of MOR mRNA and protein levels was found in cells transfected with RACK1 siRNA. In summary, this study reports a unique role of RACK1 physically interacting with PCBP1 and regulating the human MOR gene expression.

Introduction

Opioids like morphine have a long history as analgesic agents for pain management. The analgesic effect of opioids is mainly mediated through binding with opioid receptors located in the brain and the spinal cord (Kanjhan, 1995). In addition to inducing an analgesic effect, opioids are also known to exhibit undesirable side effects which are the limiting factors of their therapeutic use in chronic pain management (Dietis et al., 2011). Opioid receptors belong to the G protein-coupled receptor (GPCR) family, characterized by the presence of seven transmembrane domains, where receptor agonists activate signal transduction pathways which ultimately mediate downstream cellular responses (Simonds, 1988). GPCRs interact with ligands and modulate signal transduction processes using second messengers such as cAMP, inositol phosphate and calcium which ultimately regulate the cellular responses (Ferguson et al., 1996). Many GPCRs are known to be involved in diseases and are targeted therapeutically by modern drugs (Yeagle and Albert, 2007).

The opioid receptors are classified into three major types, μ, δ and κ according to pharmacological studies and molecular cloning (Kieffer et al., 1992, Chen et al., 1993). Upon ligand binding all three receptors get activated and affect signaling pathways which mediate functions and pharmacological effects of opioids (Kieffer and Gaveriaux-Ruff, 2002). The three opioid receptors are encoded by three different genes, yet they have high degree of sequence homology and each type of receptor has several splice variants as a result of differential mRNA processing (Knapp et al., 1995, Wei and Loh, 2011).

Mu opioid receptor (MOR) gene and structural characteristics

The morphine effect is mediated by the μ opioid receptor in the central nervous system. Binding of morphine to the μ opioid receptor is a critical step in the development of physical

dependence (Gharagozlou et al., 2003). The μ opioid receptor plays a critical role in morphine-induced analgesia, tolerance and dependence as seen from the pharmacological study and analysis of the MOR knockout mice (Narita et al., 1999). The μ opioid receptor was cloned by several groups (Kieffer, 1992, Evans, 1992, Chen et al., 1993). The cDNA clone termed MOR-1, is composed of 4 exons and its seven transmembrane domains are encoded by the first 3 exons and the 4^{th} exon encodes the last 12 amino acids of the intracellular C-terminus of the receptor. Two of the splice variants MOR-1A, and MOR-1B of the cloned μ opioid receptor have been studied and these variants show the same selectivity for the ligand (Bare et al., 1994, Pasternak, 2005). The MOR-1 gene has two different promoters, distal and proximal, located within 1 kilobase upstream of the translational start site (ATG) (Ko et al., 1997). Transcription is initiated at the distal promoter from a single transcription initiation site, located 794 bp upstream of the translation start site. The proximal promoter initiates the μ opioid receptor transcription from the four major transcription initiation sites located in a 291 to 268 bp region upstream of the translation start site (Min et al., 1994).

There are several elements that are responsible for the regulation of MOR expression, by targeting the promoter activities; I) the proximal promoter is regulated by the cis acting elements, II) trans-acting factors including a neuron-restrictive silencer element, III) an activator protein-α-like element, IV) specificity protein (Sp) binding sites (Ko et al., 1997, Ko et al., 2003, Kim et al., 2005). Using yeast one-hybrid screening system, poly (C) binding protein 1 (PCBP1) was identified as a single stranded DNA (ssDNA) binding protein which binds to the single stranded (ss) polypyrimidine (PPy) element of the MOR proximal promoter and regulates the MOR gene expression (Ko and Loh, 2005).

The K Homology (KH) Domain superfamily and PCBPs

PCBP1 belongs to the KH domain superfamily (Krecic and Swanson, 1999). In archaea, bacteria and eukarya, KH domain is found in several proteins that share similar structural features (Makeyev and Liebhaber, 2002). The KH motif is comprised of approximately 70 amino acids (Valverde et al., 2008). Initial studies identifying the characteristics of the KH domain have shown that it comprises of 45 amino acid motifs and subsequent studies have defined a more extensive 68-72 residue KH maxi domain (Siomi et al., 1994, Musco et al., 1996). Nuclear magnetic resonance (NMR) spectroscopic and X-ray crystallographic analysis of a number of KH domains have revealed that the initially described 45 residue core is configured as a *βααβ* unit forming a secondary structure (Musco et al., 1996, Baber et al., 1999, Wimberly et al., 2000). A variable linker region connecting the multiple domains in hnRNP proteins is one of the most prevalent features of the protein. The presence of the RRM (RNA Recognition Motif) characterized by a *β1-α1-β2-β3-α2-β4* structure along with two highly degenerate ribonucleoprotein (RNP) consensus sequences, RNP-1 and RNP-2 are characteristic to hnRNPs (Hoffman et al., 1991).

PCBP1 is a protein approximately 38 kDa in size and comprises of two subsets in mammalian cells; hnRNP K/J and the αCP proteins (Matunis et al., 1992, Makeyev and Liebhaber, 2002, Rivera et al., 2004, Malik et al., 2006). The αCPs are encoded at four loci, with additional isoforms generated via alternative splicing. αCP-1 and αCP-2 are well studied and are alternatively referred to as PCBP1 and PCBP2 or hnRNP-E1 and hnRNP-E2 (Makeyev and Liebhaber, 2002). PCBPs are defined by their KH structure and their ability to recognize poly

(C) rich regions in DNA and RNA (Ostareck-lederer et al., 1998, Ko et al., 2003, Malik et al., 2006). There are five PCBP loci: HNRNPK and PCBP1, 2, 3, and 4 (Makeyev and Liebhaber, 2002). PCBP1 contains three KH domains, two KH domains grouped at N terminal while the third domain is located at the C terminal. The three domains are separated by an intervening region of variable length (Makeyev and Liebhaber, 2002, Choi et al., 2005). The variable region contains a nuclear localization signal (NLS) that allows shuttling of PCBP1 between the nucleus and the cytoplasm (Berry et al., 2006). The ability of PCBPs to recognize and bind poly (C) rich DNA and RNA sequences via their KH domains is critical for their function in mammalian cells (Malik et al., 2006). The triple KH domain structure common to all PCBPs does not dominate their capacity to bind to poly (C) regions. There are several proteins like the Nova-1 and Nova-2 that also share the triple KH domain structure and exhibit different specificity to bind to the poly (C) regions (Yang et al., 1998). Hence, PCBPs are defined by the presence of triple KH domains and their capacity to bind to poly (C) regions.

Adapted from Malik et al., 2006

Figure: 1 Schematic representation of PCBP1 and the three KH domains

PCBP1 contains three KH domains, two of the KH domains located at the N terminal and one KH domain is located at the C terminal. All the three domains are separated with an intervening region of variable length; within this variable region is the nuclear localization signal.

Role of PCBPs in biological processes

The structural diversity among PCBPs plays an important role in the different functions these proteins mediate. Members of the KH domain superfamily have been shown to be involved in various biological processes from mRNA stabilization to transcriptional and translational regulation (Kiledjian et al., 1997, Choi et al., 2009). Studies have shown that PCBP1 regulates the MOR gene expression by binding to a single stranded element located in the proximal promoter (Malik et al., 2006, Rivera-Gines et al., 2006). PCBP1 also participates in regulation of eIF4E and the androgen receptor (Lynch et al., 2005, Cloke et al., 2010). In HepG2 cells PCBP1 acts a negative regulator of hyaluronic acid receptor (CD44) variants and loss of PCBP1 in human hepatic tumor contributes to the formation of a metastatic phenotype (Zhang et al., 2010). In addition, hnRNP K and PCBP1/2 are involved in the transcriptional silencing of 15-lipoxygenase (LOX) mRNA expression, shown in Fig. 2B. The translational silencing of LOX mRNA is associated with formation of a RNP complex at a conserved CU-rich repeat motif called DICE (differentiation control element) located within the 3′ UTR. In this function as a transcriptional regulator, hnRNP K and PCBP1 proteins block the assembly of 60S and 40S ribosome units necessary for the complex at the initiation site AUG (Ostareck-lederer et al., 1998).

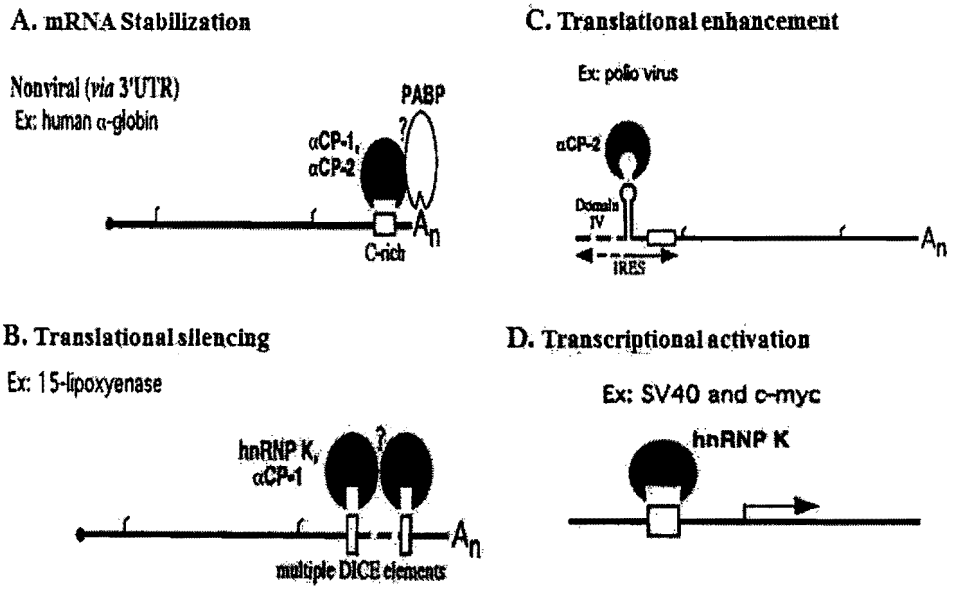
PCBPs have been associated in a wide range of posttranscriptional controls; one of the studies has shown PCBP1 to be involved in mRNA stabilization of human α-globulin as shown in Fig. 2A (Chkheidze et al., 1999). Binding of PCBP2 to the tyrosine hydroxylase (TH) mRNA 3′-UTR domain has been shown to regulate the interaction with its cognate polypyrimidine binding site. This increased interaction plays a role in the activation of TH mRNA translation by cyclic AMP (cAMP) in dopaminergic neurons (Xu et al., 2009). In cirrhotic liver, the hepatic

stellate cells (HSC) are the primary cells responsible for the dramatic increase in the synthesis of type I collagen. PCBP1 is involved in the activation and stabilization of collagen α-1 mRNA by binding to the C-rich sequence in its 3′ UTR region (Stefanovic et al., 1997).

In addition to its role in transcriptional and posttranslational regulation, PCBP1 also acts as translational enhancer where PCBP1 and PCBP2 interacts with picornaviral RNA structures and are known to regulate the viral genome translation and replication as shown in Fig. 2C (Zhang et al., 2007). The formation of an RNP complex containing the polio virus protein requires PCBP to promote RNA replication, suggesting a different role of PCBP (Andino et al., 1990). In HeLa cell extracts binding of PCBP2 to stem-loop IV within the central region of the internal ribosome entry site (IRES) is essential for efficient poliovirus translation (Blyn et al., 1996; Gamarnik and Andino, 1997). In addition to their roles in mRNA stability and translational controls, the PCBPs appear to have diverse functions in the regulation of transcriptional activation. hnRNP K has a specific binding site on the SV40 early promoter (Gaillard et al.,1994) and in the pyrimidine-rich strand of the CT element in the promoter of human *c-myc* gene, as shown in Fig. 2D (Tomonaga and Levens, 1996).

A study has shown that PCBP3 functions as a repressor, dependent on binding to single-stranded and double-stranded poly (C) sequences (Kang et al., 2012). PCBP4 also known as MCG10 can suppress cell proliferation by inducing apoptosis and cell cycle arrest (Zhu and Chen, 2000). The structural variability of PCBP isoforms can contribute to the multiplicity of reported protein-protein interactions. The above reported functional roles of a protein can also be affected by other proteins in its environment and which may be mediated through variable protein-protein interactions. These roles of PCBPs could be mediated via protein interactions

with several other proteins, hence identifying these interactions would benefit our underastanding of the effect they have on downstream cellular processes.



Adapted from Makeyev and Liebhaber, 2002

Figure: 2 Examples of functions mediated by the PCBPs.

Four distinct examples of PCBP functions are shown in the above figure. A specific example is given for each of these indicated functions and described in the text.

Known PCBP interacting proteins

Yeast two hybrid experiments have shown that hnRNPK and PCPBs interact with different proteins. hnRNPK binds to the far upstream sequence element (FUSE) of the human *c-myc* gene causing alterations in the DNA resulting in topology changes in the gene expression (Michelotti et al., 1996). hnRNPK is known to dimerize and bind to several proteins from the Src family of tyrosine kinases and with protein kinase C (Tomonaga and Levens, 1995, Schullery et al., 1999). Using the yeast two hybrid system, αCP-2 has been shown to interact with Y-box-binding protein, splicing 9G8, and filamin (Funke et al., 1996, Kim et al., 2000, Gamarnik and Andino, 1997, Kim et al., 2000). The N-terminal of αCP-2 including two exons after the second KH domain is required for both homo-dimerization and interaction with hnRNP I, K, and L (Kim et al., 2000).

General importance of protein-protein interactions

In order to understand the multifunctional role of PCBP1, it is important to study protein interactions between PCBP1 and its interacting protein partners. Within a cell, proteins interact amongst each other, phospholipids, metabolites and nucleic acids via different domains. Structural differences in different proteins play a vital role in mediating their interactions and the downstream cellular effects of the interaction complex. Studying how the interaction takes place *in vivo* will help us understand how various intracellular processes are regulated.

Several examples of how various protein interactions regulate biological processes are explained below. During mitosis, appropriate assembly and constriction of the acto-myosin based ring is important for the final separation of the two daughter cells which is coordinated by

the small GTPase, Rho. This contractile ring assembly requires the physical interaction of structural proteins like microtubules of the central spindle, motor proteins and Rho activators (Ebrahimi and Gregory, 2011). The Lin, Isl & Mec (LIM) are protein structural domains with two characteristic zinc finger motifs, these LIM domains interact via its zinc finger motifs with proteins within the nucleus, cytoplasm and can shuttle between the compartments. In one of the nuclear LIM proteins, the LIM homeodomain protein is known to regulate functions in cell lineage determination and pattern formation during animal development (Dawid et al., 1998). The Src homology 2 (SH2) and Src homology 3 (SH3) domains belong to the Src-homology (SH) domain family and are involved in molecular interactions and intracellular signal transduction processes. The SH2 domain binds tightly to phosphorylated tyrosine residues and SH3 domain mediates its protein-protein interactions through recognition of specific proline-rich sequences (Pawson et al., 2001).

Another important protein is the insulin receptor (IR); with the presence of two characteristic domains that dominate its interaction with other protein partners. Insulin receptor substrate (IRS-1) is responsible for transduction of insulin dependent signals by regulation of various processes like cellular growth and uptake of glucose. The presence of a pleckstrin homology (PH) domain at the N-terminal region followed by a phosphotyrosine binding (PTB) domain ensures selective substrate recognition and phosphorylation of the activated IR (Morris et al., 1996). IRs possess multiple tyrosine phosphorylation motifs, located at the C-terminal which serve as the docking site for the SH2 domain containing signaling molecules, such as phosphatidylinositol 3-kinase (PI 3-kinase), Grb-2 adaptor protein, and SHP2 (SH2 containing phosphatase 2) tyrosine phosphatase, which in turn stimulates the activation of biochemical cascades that promotes metabolic and cellular response to insulin (Farhang-Fallah et al., 2002).

Thus, protein domains are crucial players in the protein interactions mediating cellular signal transduction.

Rationale of this study

PCBP1 plays a vital role in various biological processes, thus it is important to understand how this protein mediates its functions. The multi-functionality of PCBP1 can be mediated through protein-protein interactions, determining how it interacts with other proteins will help understand its functional roles. The aim of this study is to identify PCBP1 interacting protein from the human brain cDNA library, characterize the interaction and study the functional effect of this interaction in the neuronal NMB cells.

Materials and Methods

Culturing neuroblastoma cells

Approximately 10^6 neuroblastoma (NMB) cells (Baumhaker et al., 1994) were grown in T75 flasks with Roswell Park Memorial Institute Medium (RPMI) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum along with 0.1% penicillin and streptomycin and maintained in an incubator at 37 °C with 5% CO_2.

Lowry assay

Using bovine serum albumin (BSA) as a standard, protein concentrations were determined using a Lowry assay. Cellular lysates were incubated at room temperature (RT) with solution C (1.8 mM Na_2CO_3, 98 mM NaOH, 0.95 mM sodium potassium tartrate, and 0.4 mM $CuSO_4.5H_2O$) for 10 minutes and then further incubated with 1 N Folin Ciocalteu's Phenol reagent for 30 minutes. The absorbance of each sample was measured at 660 nm using a spectrophotometer. Concentration of the cellular lysates was determined using a standard curve.

Nuclear extract preparation

Nucleoplasmic extracts were prepared from cells using the method previously described (Lin et al., 2008). Briefly, cells were seeded at density of approximately 10^6 cells in a petri dish, once confluency was reached; cells were then harvested using 0.1% trypsin and washed with phosphate buffered saline (PBS). The following steps were performed at 4 °C. Cells were resuspended in sucrose buffer (0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol (DTT), 0.5 mM PMSF and 0.5% NP-40 detergent). The pellet containing nuclei was collected by microcentrifugation at 500 x g, and

washed with sucrose buffer without NP-40. The nuclei were resuspended in low salt buffer (20 mM HEPES, pH 7.9, 25 % glycerol, 0.02 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF), then lysed by gently mixing with high salt buffer (20 mM HEPES, pH 7.9, 25 % glycerol, 0.8 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1% NP-40 and 0.5 mM PMSF), followed by diluent (2.5 volume of 25 mM HEPES, pH 7.6, 25 % glycerol, 0.1 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF). The chromatin DNA and its associated proteins were removed by microcentrifugation at 13,690 x g, and the supernatant which contained nucleoplasmic proteins was then ready for use.

Co-immunoprecipitation assay

Co-immunoprecipitation of endogenous RACK1 and PCBP1 was performed using untransfected NMB cells. Cells were grown in T75 flasks in RPMI medium, upon reaching 95% confluence approximately 10^6 cells were washed with serum free RPMI media, harvested with PBS -EDTA trypsin and pelleted by centrifuging at 1000 rpm for 5 minutes. Harvested cells were resuspended in 500 ml of water and lysed with 1% SDS, along with protease inhibitors phenylmethylsulfonyl fluoride (PMSF) 1 mM, pepstatin 10 μg/mL, aprotinin 1 μg/mL, and sodium vanadate at 1 μM. The protein concentration was determined by a Lowry protein assay. Cell lysate was pre-cleared with 20 μg of IgG beads for 30 minutes on a rotating platform at 4 °C. After incubation the cell extracts were centrifuged at 2500 rpm for 30 seconds. The supernatant was incubated overnight with 2 μg of anti-PCBP1 antibody or preimmune IgG serum (negative control) on a rotating platform at 4 °C. After incubation 20 μl of IgG agarose beads prewashed and resuspended in water (1:1 ratio) were added and incubated for 2 hours on rotating platform at 4 °C. The cell extracts were washed three times with RIPA buffer (1% NP40, 0.5% sodium deoxycholate, in PBS) and then separated by SDS-PAGE, described below in detail.

SDS PAGE and Western blot analysis

Samples were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) at 150 Volts for 2 hours at RT and then transferred to polyvinylidene difluoride membrane (PVDF) (GE healthcare, WI). After a series of washes in 0.1% and 0.3% TTBS (Tween-20 Tris Buffered Saline), the membrane was probed with antibodies for RACK1 and PCBP1 (Santa Cruz Biotechnology, CA). The signals for RACK1 (32 kDa) and PCBP1 (38 kDa) were detected using an enhanced chemiluminescence detection system (GE Healthcare, WI). The images were captured using Molecular Dynamic imager system (GE Healthcare, WI). Antibodies used for Western blotting are described in the table below.

Antibody	Dilutions	Species raised in	Source
RACK1	1:500	Mouse	Santa Cruz Biotechnology
PCBP1	1:2000	Goat	Santa Cruz Biotechnology

Table 1: List of primary antibodies used for Western blotting and antibody labeling experiments.

Immunofluorescence and confocal microscopy

Cells were grown on coverslips at density of 10^6 per well and fixed with 4% formaldehyde freshly prepared from paraformaldehyde. The cells were permeabilized using 0.3% Triton X-100 (Sigma Aldrich, MO). Cells were then incubated with a blocking solution containing 2% BSA and 0.3% Triton X-100 in PBS for 10 minutes at RT. After blocking, cells were incubated overnight with primary antibody for RACK1 at 4 °C (dilution 1:500) (Santa Cruz Biotechnology, CA) and then with Cy3 conjugated secondary antibody (dilution 1:100) (Jackson Immunoresearch, PA), for 2 hours at RT. The cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Invitrogen, CA). Coverslip with cells was placed on the slide containing anti-fade reagent (Vector Shield, CA) and sealed with nail polish. To check the specificity of fluorescent secondary antibody, cells were stained using the same protocol without the addition of the primary antibody. To check the specificity of primary antibody, experiments were repeated with RACK1 monoclonal as well as polyclonal antibodies.

Confocal microscopy

Above prepared slides with cells were imaged using an Olympus FLUOVIEW-1000 confocal laser scanning microscope. Cells on the slides were initially focused using the wide-field fluorescence imaging mode at 10X. After locating the cells, cells were imaged using the 40X and or 60X oil immersion objective lenses. Light exposure was limited in order to avoid photo-bleaching of the fluorescent dyes, the reporter dyes are summarized in Table 2. The gain setting for each detector was kept constant at 1X. Cells were viewed in X and Y dimensions first and the confocal image stacks (Z-optical sections) were collected using preset Z dimensions which covered the entire cell.

Dye	Excitation (nm)	Emission (nm)
DAPI (labeling nuclei)	358	461
Cy3 (Secondary Antibody)	554	568

Table 2: Excitation and emission wavelengths of dyes utilized in confocal microscope.

Cloning of RACK1 deletion constructs

Twelve deletion constructs were designed; six deletion constructs of RACK1 150–316 amino acids were constructed via deletion of residues from the N terminus of the fragment, sequentially deleting individual WD domains (McCahill et al., 2002). The remaining six constructs were constructed, each comprised of an individual WD domain (McCahill et al., 2002).

Construct	Primer sequence
WD1	5′-ACTGAGCAGATGACCCTT-3′
WD2-7	5′-GAGACCAACTATGGA-3′
WD3-7	5′-ACCACCACGGGCGA-3′
WD4-7	5′-GTGCAAATACACTGT-3′
WD5-7	5′-TGCAAGCTGAAGACC-3′
WD6-7	5′-AAACACCTTTACACGCTA-3′
WD 7	5′-AAGCAAGAAG-3′
WD1R	5′-ACCTTTGACTGGTCCCTAATC-3′
WD2R	5′-GCGG AGACCCTAGAGTGTATC-3′
WD3R	5′-TCTGCCGGTTGTCAGAGGAGAAGG-3′
WD4R	5′-CATACCTTGGACCGAATC-3′
WD5R	5′-CGGTACAATACCCTAGAGATC-3′
WD6R	5′-TTCATCTACAATGATCTTTCCCTCATC-3′
WD7R	5′ -TGGTAACCGTGTGCGATCCATAGTTTATC-3′

Table 3: List of primers used in PCR to generate the RACK1 constructs

Amplified inserts were cloned into the pCR2.1 vector (Invitrogen, CA) via *Eco*RI site and subcloned into pTRG vector (Agilent Technologies, CA) via *Spe*I and *Not*I site. Insert orientation was determined via restriction enzyme analysis and confirmed by DNA sequencing.

Bacterial transformation

JM109 competent cells (Invitrogen, CA) were used for bacterial transformation according to manufacturer's protocol. Briefly, cells were incubated with 50 ng of plasmid on ice for 30 min. After incubation, cells were heat shocked at 42 °C and then placed on ice for 2 min. Luria Broth (LB) media was added and cells were incubated at 37 °C with shaking at 225 rpm for 60 min. The resultant transformants were plated on LB media containing 10 mg/ml of ampicillin as selection marker and incubated overnight at 37 °C.

Small scale plasmid purification

Plasmids were extracted from cultures using a Qiaprep Miniprep Kit from Qiagen. Cultures were centrifuged and pellets were resuspended in re-suspension buffer P1 (50 mM glucose, 10 mM EDTA, pH 8.5, 25 mM Tris HCl, pH 8). Resuspended cells were lysed using lysis buffer P2 (0.2 M NaOH, 1% SDS), and then neutralized by adding neutralizing buffer N3 (5 M potassium acetate, glacial acetic acid). The plasmids were separated by centrifugation and applied to Qiaprep spin column and washed with washing buffer PB and PE. The plasmids were then eluted using elution buffer EB (10 mM Tris HCl, 1 mM EDTA, and pH 8.4).

Large scale plasmid purification

Bacterial cultures were grown overnight at 37 °C with shaking at 225 rpm. The plasmids were extracted using a Qiagen Plasmid Maxi prep Kit (Qiagen, CA). Bacterial cultures were harvested

by centrifugation at 6000 x g and the pellet was resuspended in 10 mL of re-suspension buffer P1 (50 mM glucose, 10 mM EDTA, pH 8.5, 25 mM Tris HCl, pH 8). The bacterial suspension was then lysed by adding 10 mL of lysis buffer P2 (0.2 M NaOH, 1% SDS), which was further neutralized by adding 10 mL of neutralizing buffer P3 (5 M potassium acetate, glacial acetic acid). The mixture was centrifuged at 15,000 rpm for 30 min. The supernatant was applied to a Qiagen-tip column, which was then washed with washing buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol). The plasmid was eluted using elution buffer QF (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5, 15% isopropanol), and was precipitated using isopropanol precipitation method.

Bacteria two-hybrid screening

XL1-Blue MRF′ Kan strain of competent cells was purchased from Agilent Technologies, CA. According to the manufacturer's protocol, competent cells were incubated with 1.7 µl of β-mercaptoethanol on ice for 10 min, swirling every 2 min. Individual deletion domain construct (Target) plus the bait containing full length PCBP1 were incubated with competent cell mixture and placed on ice for 30 min. Cells were subjected to heat shock reaction at 42 °C and then incubated on ice for 2 min. SOC media (20 g tryptone, 5 g of yeast extract, 0.5 g NaCl, 1 M $MgCl_2$, 1 M $MgSO_4$, 2 M glucose) was added to the cells, which were then incubated at 37 °C, with shaking at 225 rpm for 90 min. Cells were collected and washed with M9+ dropout broth (10X M9 salts, M9 media additives (0.2% glucose, 0.02 mM adenine HCL, 10X His dropout amino acid supplement, 1M $MgSO_4$, 1 mM thiamine HCL, 0.1 mM $ZnSO_4$, 0.1 mM $CaCl_2$, 0.05 mM IPTG)) and then resuspended in M9 minimal salts broth with dropout minus histidine (M9+ His-dropout broth). Cells were incubated at 37 °C with shaking at 225 rpm for 2 hours and then plated on agar plates containing the four selection markers; 0.5 ml of 25 mg/ml chloramphenicol,

0.5 ml of 12.5 mg/ml tetracycline, 2.5 ml of 1 M 3-AT (dissolved in DMSO) and 0.5 ml of 12.5 mg/ml streptomycin incubated at 37 °C. The surviving clones were further verified using X-gal screening.

RNA isolation and RT-PCR

Total RNA from the cells was extracted using Tri-reagent (Invitrogen, CA) first and then with chloroform. The mixture was subjected to centrifugation at 12000 x g for 5 min at 4 °C. The top phase containing total RNA was separated and RNA was precipitated using 100% molecular biology grade isopropanol (Sigma Aldrich, MO) at RT followed by centrifugation at 12000 x g for 15 min. The RNA pellet was washed with 70% ethanol and dissolved in Diethyl pyrocarbonate (DEPC) treated water. RNA concentration was determined by using UV spectrophotometer and calculated by formula OD 260 X 50 µg/ ml. First strand cDNA was synthesized using 5 µg of total RNA using the random primer, in the presence of 200 units of reverse transcriptase. Amplification was performed for 50 min at 37 °C and 15 min at 70 °C. PCR amplification of cDNA was performed for 1 min at 95 °C, 35 s at 68 °C, and 40 s at 72 °C for 32 cycles.

Agarose gel electrophoresis

The PCR products were analyzed by running on a 2% agarose gel containing 0.5 µg/ml of ethidium bromide, separated at 135 volts for 30 min. The gel was then imaged using Alpha imager gel doc under UV elimination (Alpha Innotech Corporation, CA)

Transient transfection and luciferase Reporter gene assay

NMB cells were seeded at densities 10^6 per well in 6-well culture plates (Corning, NY). After twenty-four hours cells were transfected using 1:2 ratio of DNA to lipofectamine reagent according to manufacturer's protocol (Invitrogen, CA). RACK1-PcDNA3 plasmid was used in three different concentrations 0.2 μg, 0.5 μg and 1μg along with Pgl3-MOR plasmid at 0.5 μg. After forty-eight hours, cells were washed and incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum. After seventy-two hours, the cells were harvested and lysed with the reporter lysis buffer (Promega, WI). The cell lysates were subjected to luciferase assay using a luciferase assay system (Promega, WI), and the activity was measured as relative light units (RLU) using a luminometer (Berthold). A Lowry assay was performed to determine concentration of protein present in the lysates and the data was normalized with the amount of protein.

SiRNA transfection and whole cell preparation

NMB cells were seeded at densities 10^6 per well in 6-well culture plates (Corning, NY) and then transfected with RACK1 siRNA using lipofectamine RNAimax according to manufacturer's protocol (Invitrogen, CA). The amount of lipofectamine was calculated using the manufacturer's recommendation using 1:1 RNA to lipofectamine ratio. RACK1 siRNA was used at three different concentrations 50 nM, 100 nM and 200 nM. After forty-eight hours, the cells were washed and incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum. After seventy-two hours, the cells were harvested using 0.1% trypsin by centrifugation at RT for 5 min at 1000 x g. The cell pellet was resuspended in 0.2 ml of 25 mM HEPES (NaCl 115 mM, $CaCl_2$

1.2 mM, $MgCl_2$, K_2HPO_4 2.4 mM, PH 7.4). The harvested cells were used for binding experiments explained in detail below.

Competition opioid binding assay

Cells were incubated for 10 min at RT with labeled [^{3}H] diprenorphine ligand at 2 nM concentration in the presence or absence of 1 μM unlabeled ligand CTAP, in 25 mM HEPES (pH 7.4). The two other receptor subtypes were blocked with subtype selective ligands (δ) DADLE (D-Ala2, D-Leu5)-Enkephalin and (κ) U50488 at concentrations of 1 μM. After incubation with ligands the reaction was terminated by filtration on GF/B filters, the filters were washed two times with 7% polyethylene glycol (PEG) in HEPES buffer precooled at 4 °C and allowed to stand with Scintiverse-BD scintillation fluid (Fisher, PA) for 1 hour at 22-25 °C before counting with a scintillation counter (Perkin Elmer, MA). A Lowry assay was performed to determine the protein concentration in the control cell lysate and siRNA transfected cell lysate. Data was normalized using the amount of protein present in the control lysate.

Statistical analysis

Values are reported as mean ± SE from at least three independent experiments. Statistical significance was determined using Student's paired t test.

Data quantification

Western blot and RT-PCR data was quantified using Image Quant software (GE Healthcare, WI).

Results

Identification of PCBP1-interacting protein using bacteria two hybrid system

Human brain cDNA library (Agilent Technologies, CA) was screened using bacteria two hybrid system to identify a PCBP1 interacting protein. Full length PCBP1 was cloned into pBT vector (PCBP-pBT) and human cDNA library was cloned in to the pTRG vector as shown in Fig. 1, both the plasmids were cotransformed and subjected to screening using high stringency media containing 3-amino-1,2,4-trizole (3-AT), streptomycin, chloramphenicol, tetracycline and streptomycin (explained in detail under materials and methods section, Page 24). One of the positive clones that grew on the media screening contained 1 kilobase of cDNA insert. The positive clone was subjected to DNA sequencing and NCBI blast analysis and was identified as RACK1.

In order to validate the interaction between RACK1 and PCBP1, RACK1 was cloned in the pTRG vector of the two hybrid system. PCBP1-pBT and RACK1-pTRG were cotransformed and were subjected to screening by ability to grow at 37 °C on high stringency media (explained in detail under materials and methods section, Page 24). All the clones subjected to high stringency screening grew on the screening media. The surviving clones under high stringency selection were reproduced and verified to validate the interaction between RACK1 and PCBP1. Thus, bacteria two hybrid screening results have showed that PCBP1 and RACK1 interact with each other.

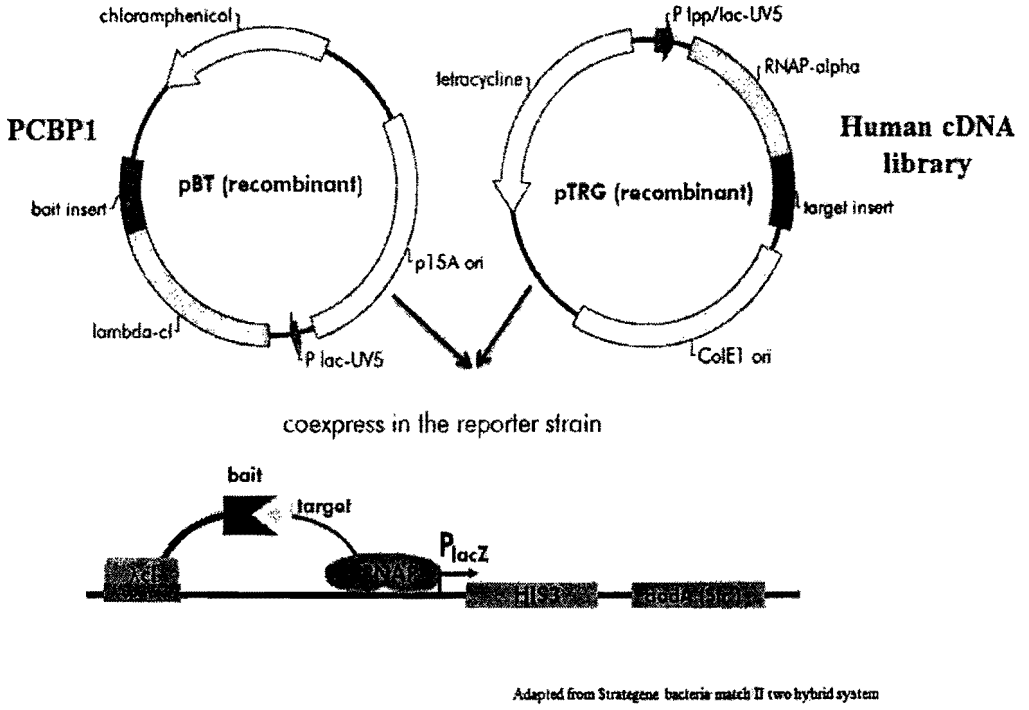


Figure 3: Screening using bacteria two hybrid system

Schematic of the bacteria two hybrid system where PCBP1 (bait) was cloned in to the pBT vector and human brain cDNA library (target) was cloned in to the pTRG vector. When the bait and target protein interact, they recruit and stabilize the binding of RNA polymerase at the promoter and activate the transcription of the *HIS3* reporter gene which allows bacterial cells to grow on the media containing 3-AT. Thus, bacteria two hybrid system helps to identify the interacting protein from a cDNA library and also helps to characterize the interaction between a known protein pair.

RACK1 physically interacts with PCBP1

In order to determine if the interaction between RACK1 and PCBP1 also occurs in the mammalian neuronal cells, the human neuroblastoma (NMB) cell model system was selected. PCBP1 is expressed endogenously in the NMB cells (Lin et al., 2008). The endogenous expression of both RACK1 and PCBP1 was determined by subjecting NMB cell lysate to Western blot analysis. As shown in Fig. 4A, in lane 1, a 38 kDa PCBP1 protein was observed. The same blot was later probed with anti-RACK1 antibody (lane 2) and a 32 kDa RACK1 protein was also observed. Thus, results of Western blot analysis confirmed expression of both RACK1 and PCBP1 in the NMB cell system.

Upon confirmation of endogenous expression of both RACK1 and PCBP1 proteins, co-immunoprecipitation assay was performed using the NMB cell lysate. An anti-PCBP1 antibody was used to immunoprecipitate PCBP1 from NMB cells prior to detection of RACK1 by Western blotting. As shown in Fig. 4B, lane 2, a 38 kDa band, corresponding to PCBP1 was observed successfully precipitating PCBP1 and absent in the control lane 1. Lane 3 consists of plain NMB lysate. The same blot was reprobed with anti-RACK1 antibody without stripping PCBP1 signal. As shown in Fig. 4C, lane 2, a 32 kDa band for RACK1 was observed in the same lane as PCBP1 and absent in the control lane, indicating that RACK1 co-immunoprecipitated with PCBP1. In summary, this data confirmed the physical interaction between RACK1 and PCBP1 in the human neuronal cells.

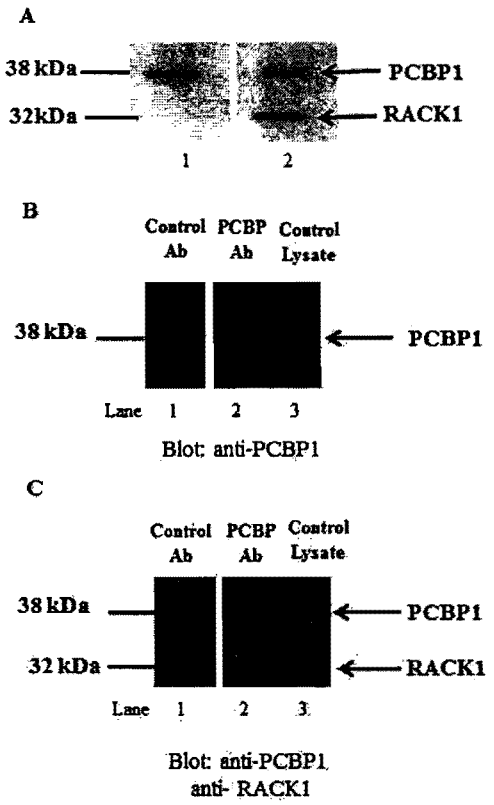


Figure 4: Co-immunoprecipitation of RACK1 and PCBP1

(A) Results from Western blot showing endogenous expression of both proteins, PCBP1 and RACK1 in the NMB cells. The whole cell lysate from NMB cells was subjected to SDS-PAGE and analyzed with anti-PCBP1 antibody showing a 38 kDa band for PCBP1 (lane 1). The same blot was probed with anti-RACK1 antibody showing a 32 kDa band for RACK1 (lane 2) without stripping off PCBP1 signal. (B) Co-immunoprecipitation of PCBP1 and RACK1, whole cell lysates from NMB cells were incubated with anti-PCBP1 Ab (lane 2) and anti- IgG (negative control lane 1). Lane 3, labeled as control lysate is whole cell lysate alone. The bound proteins were subjected to SDS-PAGE and then Western blot analysis using anti-PCBP1 antibody. A 38kDa band for PCBP1 (lane 2) was observed, indicating that PCBP1 was successfully

immunoprecipitated. (C) The same blot was reprobed with anti-RACK1 antibody without stripping the signal of PCBP1, showed that anti-PCBP1 Ab successfully precipitates RACK1 a 32 kDa band indicated (Lane 2), and the 32 kDa band was absent in the control lane (lane 1).

Intracellular localization of RACK1

The intracellular distribution of RACK1 was investigated by indirect immunofluorescence staining followed by confocal laser-scanning microscopy and Western blot analysis. Equal amounts of the whole cell lysate and the nuclear extract lysate were subjected to Western blotting and the RACK1 protein was detected using anti-RACK1 antibody. As shown in Fig. 5A, RACK1 was localized both in the nucleus and cytoplasm. The whole cell lysate contained higher amount of RACK1 protein than the nuclear extract. The quantified data in form of histograms are shown in Fig. 5B; RACK1 signal in whole cell lysate is defined as 100%. The data from the histograms showed that RACK1 is expressed at higher levels in the cytoplasm when compared to the nucleus. Cellular distribution of RACK1 was further studied using antibody labeling experiments.

Neuronal cells were labeled with anti-RACK1 antibody which was detected using Cy3-conjugated anti-IgG secondary antibody. RACK1 has a prominent cytosolic distribution with a diffuse nuclear distribution Fig. 5C (I). The nucleus was counterstained with DAPI as shown in Fig. 5C (II) and the merged images from both Cy3 and DAPI channels are shown in Fig. 5C (III). Combined results of Western blot analysis and antibody labeling experiment show that RACK1 expression levels were higher in the cytoplasm when compared to the nucleus. Thus, RACK1 localization in NMB cells was predominantly higher in the cytoplasm when compared to the nucleus.

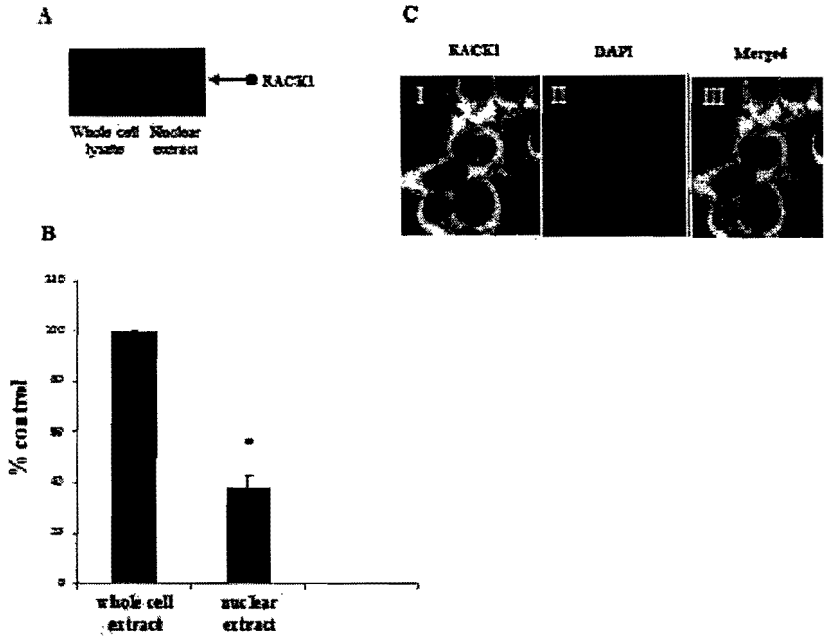


Figure 5: Intracellular localization of RACK1 in NMB cells

(A) Results from Western blot analysis of RACK1 comparing whole cell lysate and nuclear extract. A 32 kDa band for RACK1 was found in the nuclear extract, suggesting its nuclear localization. RACK1 has prominent cytosolic distribution when compared to its nuclear distribution evident from Western blot analysis. (B) Quantified histograms of three different Western blots shown here, the amount of RACK1 from whole cell lysate was defined as 100 %. The histograms are presented as mean $\pm$ SE. "*" indicates $p < 0.01$ (Student's paired t-test). (C) Immunofluorescence staining showing RACK1 distribution in the cytosol as well as nucleoplasm (C-I). Nucleus was stained with DAPI (C-II) and the merged image (C-III) shows that, RACK1 has prominent cytosolic distribution as compared to nuclear distribution.

Cellular localization of RACK1-PCBP1 interaction

The physical interaction between PCBP1 and RACK1 was confirmed by the Co-immunoprecipitation experiment shown in Fig. 3. The data from antibody labeling experiment has demonstrated that RACK1 is localized both in the nucleus and the cytoplasm Fig. 5A. The interaction between RACK1 and PCBP1 was further examined. A co-immunoprecipitation experiment using anti-PCBP1 antibody was performed to compare nuclear extract and whole cell lysate. As shown in the Fig. 6A, a 38 kDa band corresponding to PCBP1 was observed in the whole cell lysate. The same blot was probed with anti-RACK1 antibody shown in Fig. 6B lane 2; a 32 kDa band of RACK1 was also seen. Thus, the PCBP1 antibody successfully precipitated PCBP1 and its interacting protein RACK1 from the whole cell lysate. Similarly a co-immunoprecipitation experiment using the anti-PCBP1 antibody was performed using the nuclear extract as shown in the Fig. 6C. A 38 kDa band for PCBP1 was observed in the nuclear extract. The same blot was probed with anti-RACK1 antibody. Here however, the 32 kDa band for RACK1 was not observed. Results of the co-immunoprecipitation experiments thus indicate that, PCBP1 and RACK1 interact in the cytosol but this interaction does not occur within the nucleus.

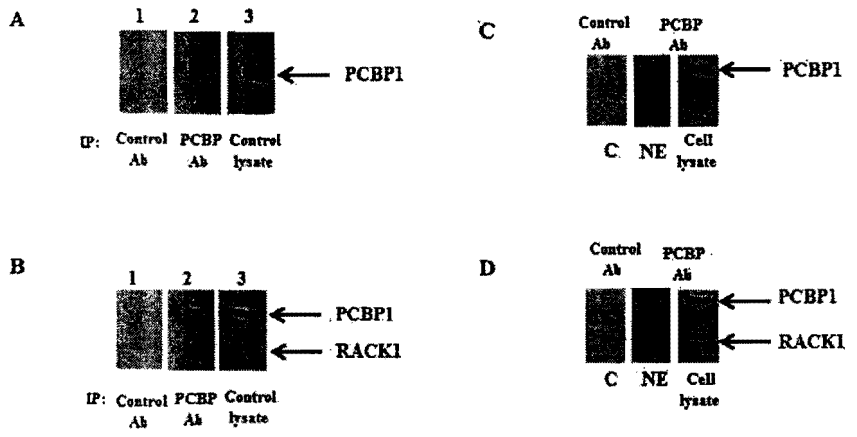


Figure 6: Co-immunoprecipitaion of RACK1 and PCBP1 using NMB lysate

(A) Results from Western blot analysis of co-immunoprecipitation assay using whole cell lysate. Whole cell lysate from NMB cells was incubated with anti-PCBP1 antibody (lane 2) and non-specific antibody (lane 1, negative control). Lane 3 labeled as control lysate is whole cell lysate alone. The Western blot analysis has shown to successfully precipitate PCBP1 (B) The same blot was probed with anti-RACK1 antibody without stripping the PCBP1 signal. The Western blot analysis shows that anti-PCBP1 antibody successfully co-precipitates RACK1 (lane 2, arrow) whereas no RACK1 was detected in the negative control IgG (lane1). (C) Nuclear extract from the NMB cells was incubated with anti-PCBP1 antibody (labeled NE) and non-specific anti-IgG antibody (labeled as C, negative control). Lane 3 labeled as cell lysate is the nuclear extract lysate alone. Western blot analysis using anti-PCBP1 antibody has shown to successfully precipitate PCBP1 from the NMB cell lysate (38 kDa band, arrow). (D) The same blot was then probed with anti-RACK1 antibody without stripping the PCBP1 signal. The Western blot

analysis has shown that anti-PCBP1 antibody did not co-precipitate RACK1 (NE) and no RACK1 was also detected in the IgG (C negative control) lane.

Minimal domain mapping of PCBP1 –RACK1 interaction

The presence of seven WD-40 repeats in RACK1 has implicated its role in protein-protein interactions (Imai et al., 2009). The domain required for the interaction between PCBP1 and RACK1 was mapped by deleting the WD domains from the N terminus of RACK1. The six RACK1 deletion plasmids were constructed using polymerase chain reaction (PCR), and PCR products were cloned into pCR2.1 cloning vector. The positive clones containing the PCR products were screened by restriction enzyme digests followed by agarose gel electrophoretic analysis (described in detail under materials and method section page 25). The plasmids containing the PCR products were then subjected to DNA sequencing. Restriction enzyme digests were run on agarose gel as shown in the Fig. 7. The predicted size PCR products for each of the six constructs are shown by an arrow. In summary, using PCR cloning method RACK1 WD domain deletion constructs were successfully generated.

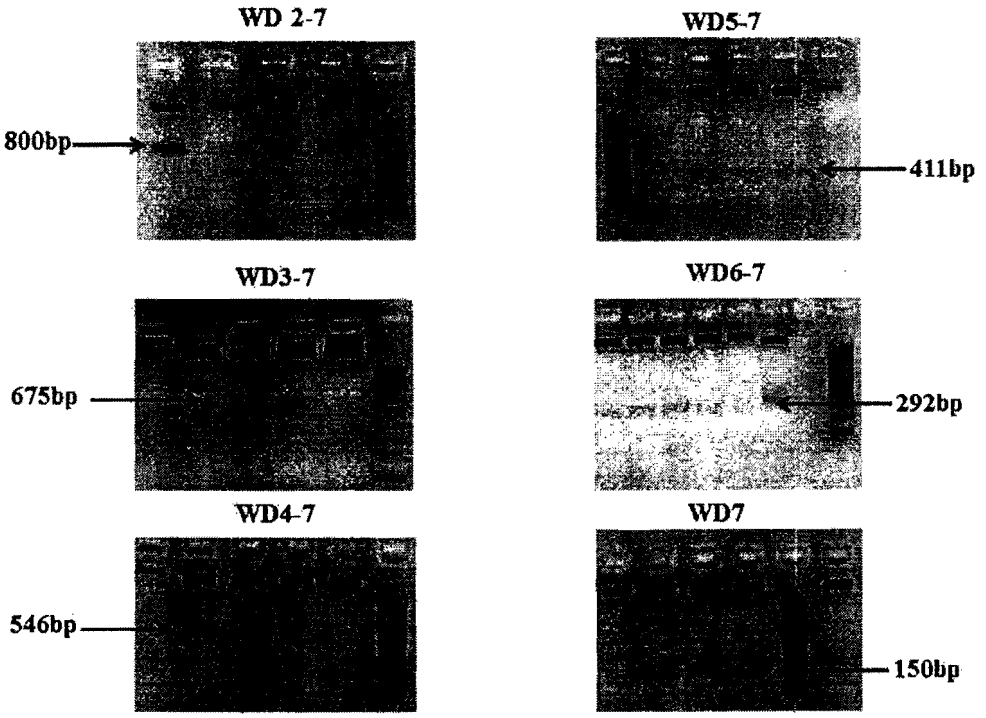


Figure 7: Cloning of RACK1 WD domain deletion construct by PCR

Agarose gel electrophoresis of cloned sequentially truncated six RACK1 (WD2-7, 3-7, 4-7, 5-7, 6-7 and 7) constructs into the pCR2.1 vector. The PCR products cloned into the pCR2.1 vector were screened using *Eco*RI restriction enzyme. The digested products were run on 2% agarose gel containing 0.5 μg/ml of ethidium bromide. The insert band of correct size is indicated by an arrow and the band seen at much higher size represents the cut pCR2.1 vector. On each gel picture above, 100 bp ladder was used and the positive clones were confirmed by DNA sequencing.

Subcloning of the WD deletion domains into the Bacteria two-hybrid vector system

The six sequentially truncated RACK1 WD domain constructs in pCR 2.1 vector were grown and then subcloned into the pTRG vector of the bacteria two-hybrid system. The WD deletion constructs were cloned into the *Not*I and *Spe*I site, via restriction enzyme digestion. The digested product was ligated with the pTRG vector, transformed in the bacterial cells and further screened. To ensure positive clones contained the specific WD domain insert, *Not*I and *Spe*I digests were performed followed by agarose gel electrophoresis. As shown in the Fig. 6, an insert band of correct size is indicated by an arrow and the band seen at much higher size represents the cut pTRG vector. The positive clones were sequenced to check for sequence specificity. In summary, RACK1 truncated constructs were successfully subcloned using pTRG vector into the bacteria two hybrid system.

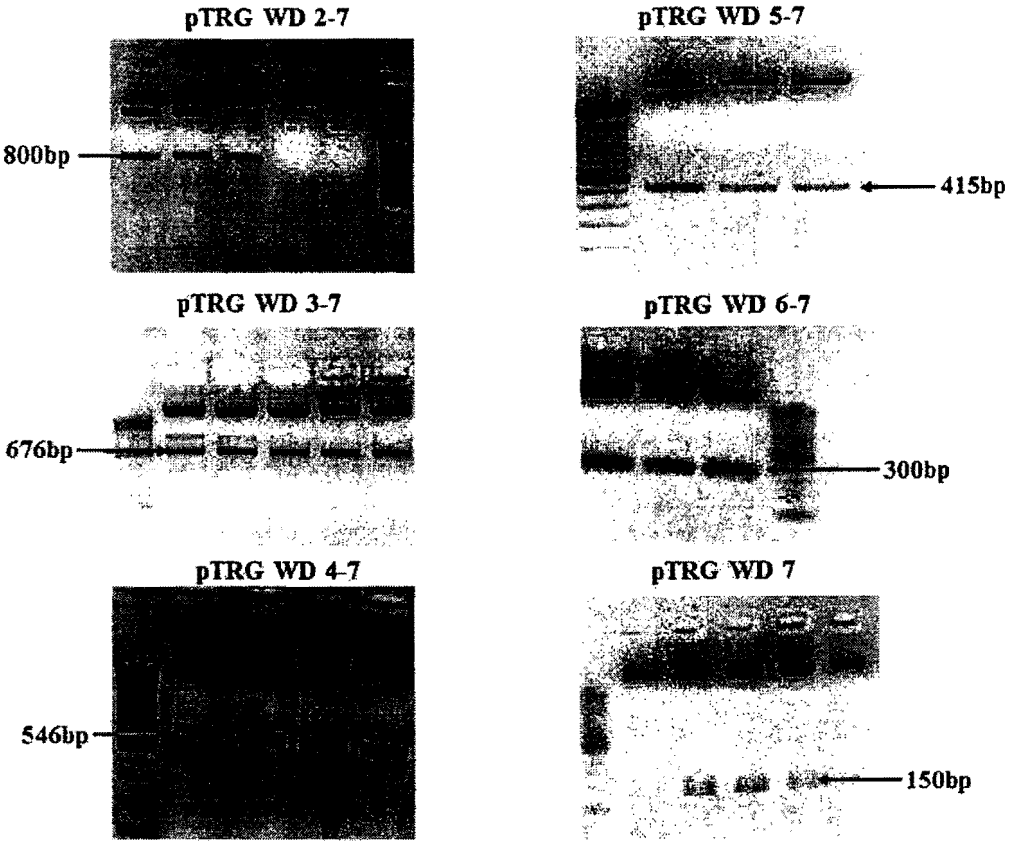


Figure 8: Subcloning of RACK1 WD deletion constructs into the pTRG vector

Agarose gel electrophoresis of subcloned RACK1 truncated constructs into pTRG vector. PCR products subcloned into the pTRG vector were screened using *Not*I and *Spe*I restriction enzymes. The digested products were run on 2% agarose gel containing 0.5 µg/ml of ethidium bromide. The insert band of correct size is indicated by an arrow and the band seen at much higher size represents the cut pTRG vector. On each gel picture above, 100 bp ladder was used and the positive clones were confirmed by DNA sequencing.

Mapping the interaction site using the bacteria II hybrid system

The pTRG subcloned RACK1 truncated constructs were used to test for their ability to interact with full length PCBP1, using the bacteria two-hybrid system screening with three selective markers, chloramphenicol, tetracycline and 3-AT. Colonies that grew on media containing three markers were streaked onto higher stringency media containing four selective markers: 3-AT, chloramphenicol, tetracycline and streptomycin (described in detail under materials and method section page 24). These selection media were selective for both the plasmids as well as for the interaction between the proteins encoded by both the plasmids. As shown in the Fig. 9, among all the constructs of RACK1, the strongest interaction between PCBP1 and RACK1 was observed with full length RACK1. Deletion of WD1 domain (WD 2-7) and WD 2 (WD 3-7) resulted in decrease in the interaction and further deletion of WD4 (WD 5-7) showed a significant decrease in the interaction. Deletion of WD1-5 (WD 6-7) has shown to have no significant effect on interaction as compared to that of the WD 5-7. Importantly, WD7 solely encoding the seventh WD-40 domain showed strongest interaction with PCBP1, comparable to that of full length RACK1. These results implicate that the WD7 domain is the key domain which bestows RACK1 the ability to interact with PCBP1. To ensure that only WD 7 is the key domain responsible for the RACK1-PCBP1 interaction and the interaction is not dependent on the other WD domains of RACK1, six clones containing individual WD domains were constructed and tested explained in the Fig.11.

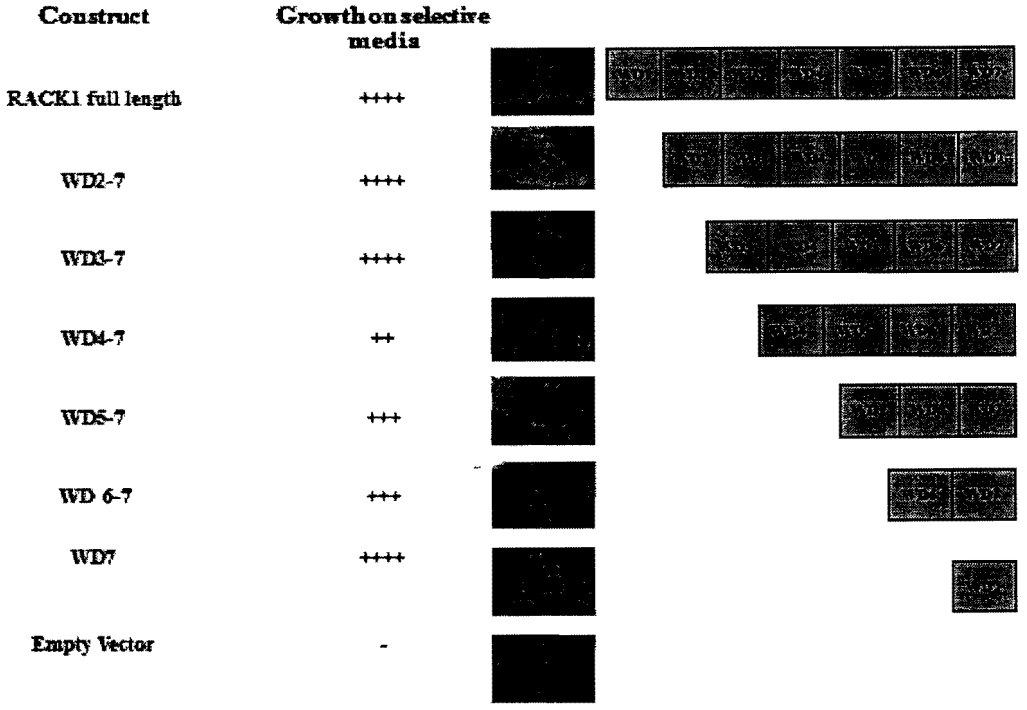


Figure 9: Minimal Domain Mapping of PCBP1 –RACK1 interaction

Listed above are deletion constructs names under construct column and corresponding domains on the right. RACK1 deletion constructs were cotransformed with full length PCBP1 using the bacteria two hybrid system. Resultant transformants were subjected to selection media containing tetracycline, chloramphenicol, 3-AT and streptomycin. Empty vector (pBT) was used as a negative control. The interaction between PCBP1 and RACK1 was assessed by 'ability to grow' (indicated by + sign), with the growth of full length RACK1 and PCBP1 indicated as (++++). The growth results shown here represent three independent experiments. Among all the six RACK1 constructs, WD7 has shown the strongest growth and thus ability to interact with PCBP1.

Cloning of individual WD domain of RACK1

Six RACK1 constructs containing each of the domains, WD1-6 were cloned using PCR cloning method. PCR was performed and PCR products were ligated with pCR2.1 vector, transformed in the bacterial cells and further screened. The positive clones containing the PCR products were screened by *Eco*RI restriction enzyme digests followed by gel electrophoresis analysis shown in the Fig. 10. The plasmids containing the PCR products were then subjected to DNA sequencing. PCR products for each of the six constructs below are indicated by an arrow and the band seen at much higher size is the cut pCR2.1 vector. In summary, using PCR cloning, individual WD domain constructs of RACK1 were successfully generated.

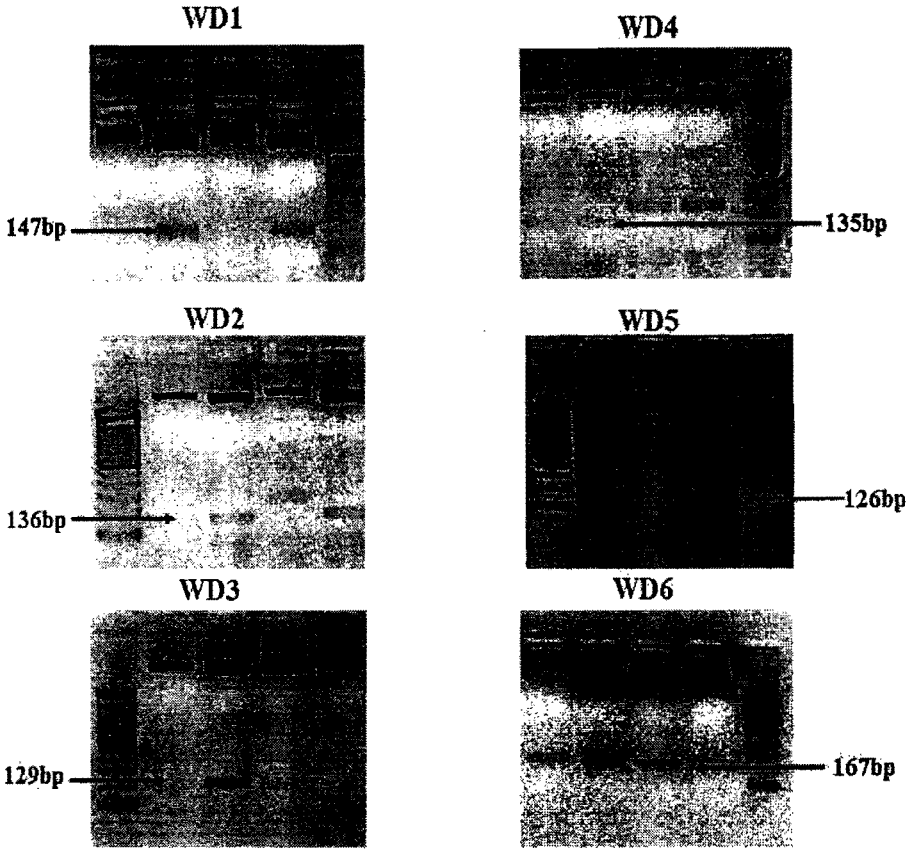


Figure 10: Cloning of individual WD domains of RACK1

Agarose gel electrophoresis of six RACK1 (WD1, WD2, WD3, WD4, WD5 and WD6) deletion constructs cloned in the pCR 2.1 vector. The constructs were generated by PCR and products cloned into the pCR2.1 vector were screened using *Eco*RI restriction enzyme. The digested products were run on 2% agarose gel containing 0.5 μg/ml of ethidium bromide. The insert band of correct size is indicated by an arrow and the band seen at much higher size represents the cut pCR2.1 vector. On each gel picture above, 100 bp ladder was used and the positive clones were confirmed by DNA sequencing.

Subcloning of Individual WD domains in the pTRG vector

The six WD constructs in the pCR2.1 vector were grown and subcloned into the pTRG vector of the bacteria two-hybrid system. The WD deletion constructs were cloned into the *Not*I and *Spe*I sites, via restriction enzyme digestion. The digested product was ligated with the pTRG vector, transformed in the bacterial cells and screened. To ensure a positive clone contained the respective WD domain insert, *Not*I and *Spe*I digests were performed followed by agarose gel electrophoresis. As shown in Fig. 11, an insert band of correct size is indicated by an arrow and a band seen at much higher size represents the cut pTRG vector. The positive clones were subjected to DNA sequencing. In summary, using the bacteria two hybrid system RACK1 domain deletion constructs were successfully cloned in to the pTRG vector.

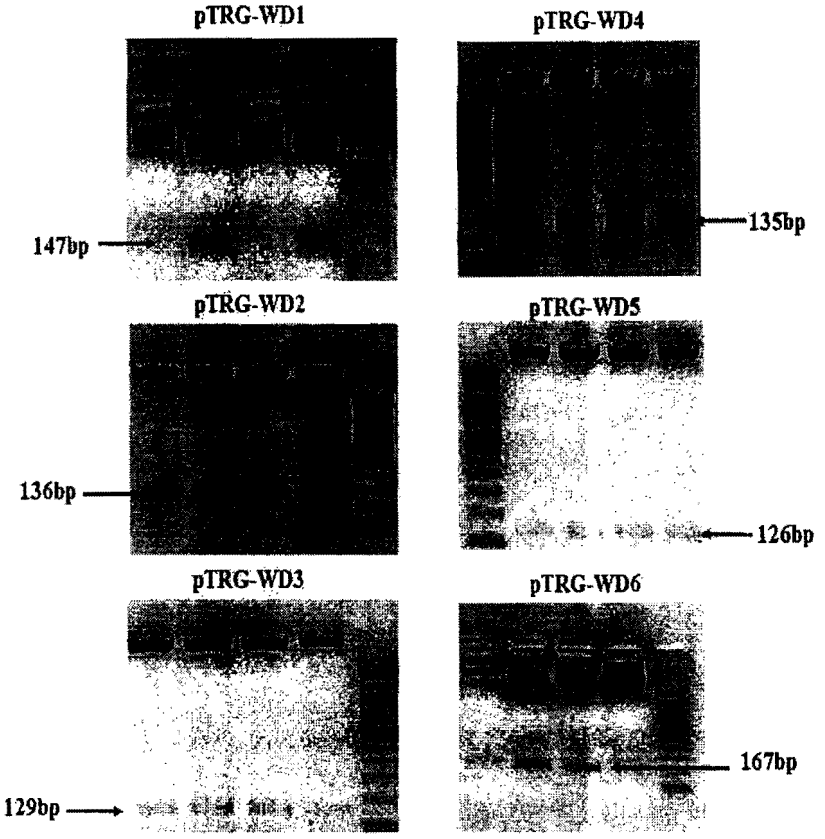


Figure 11: Subcloning of Individual WD domains in the pTRG vector

Agarose gel electrophoresis of subcloned RACK1 individual domain constructs into pTRG vector. PCR products cloned into the pTRG vector were screened using *Not*I and *Spe*I restriction enzymes. The digested products were run on 2% agarose gel containing 0.5 µg/ml of ethidium bromide. The insert band of correct size is indicated by an arrow and the band seen at much higher size represents the cut pTRG vector. On each gel picture above, 100 bp ladder was used and the positive clones were confirmed by DNA sequencing.

Testing the individual RACK1 domains for interaction with PCBP1

Using the two-hybrid system, each plasmid containing individual WD domain was co-transformed with pBT-PCBP1 plasmid and screened under the high stringency selection media (described in details under materials and methods section page 24). The interaction capacity of the WD domains was evaluated by growth rate on screening media as compared to the full length RACK1. The interaction with full length RACK1 was defined as 100% and was the strongest among all constructs. As shown in Fig. 12, in addition to the WD7 domain, the WD2 and WD3 domains of RACK1 also displayed some ability to interact with PCBP1, but the growth was much reduced when compared to that of the WD7 domain, indicating a weaker interaction. The WD6 domain interacted very weakly and the remaining domains (WD1, 4 and 5) did not show ability to interact with PCBP1. Among all the domains, WD 7 alone has shown to possess the strongest ability to interact with PCBP1. Combined results from the screening of deletion WD constructs (Fig. 9) and the screening of the individual WD constructs (Fig. 12) have demonstrated that the WD7 domain of RACK1 is crucial for the interaction with RACK1 and PCBP1.

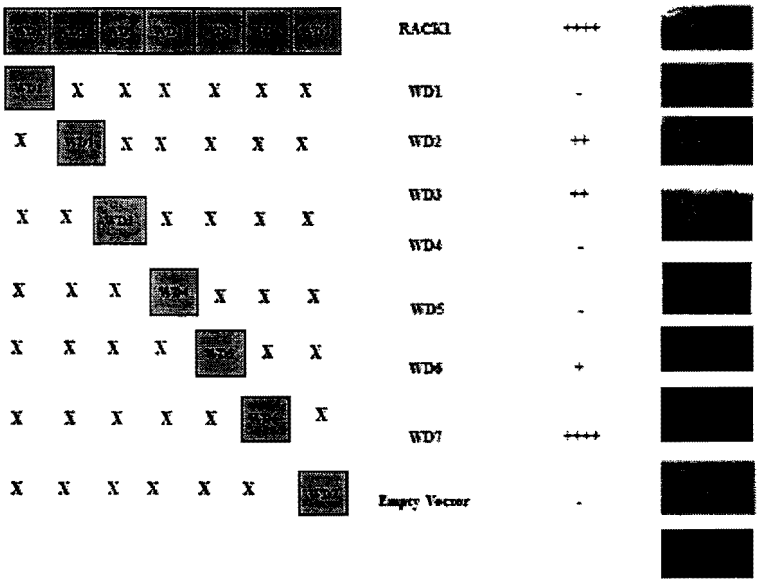


Figure 12: Testing the individual RACK1 domains for interaction with PCBP1

Listed above are deletion constructs names under construct column and corresponding domains on the right. RACK1 deletion constructs were cotransformed with full length PCBP1 using the bacteria two hybrid system. Resultant transformants were subjected to selection media containing tetracycline, chloramphenicol, 3-AT and streptomycin. Empty vector (pBT) was used as a negative control. The interaction between PCBP1 and RACK1 was assessed by 'ability to grow' (indicated by + sign), with the growth of full length RACK1 and PCBP1 indicated as (++++). The growth results shown here represent three independent experiments. Among all the 7 RACK1 constructs, WD7 has shown the strongest growth and thus ability to interact with PCBP1.

RACK 1 regulates the Mu opioid receptor (MOR) promoter activity

The data from co-immunoprecipitation experiments has shown that PCBP1 and RACK1 interact endogenously in the NMB cell system (Fig. 3). The functional effect of this interaction was further studied. Studies have shown PCBP1 to regulate the MOR expression by binding to ssDNA element located in the proximal promoter (Malik et al., 2006). Therefore, the effect of RACK1 overexpression on human MOR (hMOR) gene expression in NMB cells was investigated. The RACK1 cDNA was cloned into a mammalian expression vector pcDNA3 resulting in the pcDNA3-RACK1 plasmid. Luciferase reporter gene assay was then performed by cotransfection of 0.2, 0.5, and 1.0 μg concentrations of pcDNA3-RACK1 plasmid. The blank vector (pcDNA3) was used as a control, along with the luciferase reporter plasmid p357 containing the hMOR promoter (Cook et al., 2010). The luciferase assay was performed and data was normalized using the amount of the total protein present in each sample. The normalized activity of blank vector was defined as 100%. As shown in the Fig. 13, the reporter activity of hMOR decreased as the amount of RACK1 overexpression increased. The quantified data has shown a 50% decrease in hMOR promoter activity in samples transfected with 1 μg of RACK1 plasmid when compared to control. These findings suggest that the interaction between RACK1 and PCBP1 has a functional effect on the hMOR promoter activity, which may influence the MOR gene expression.

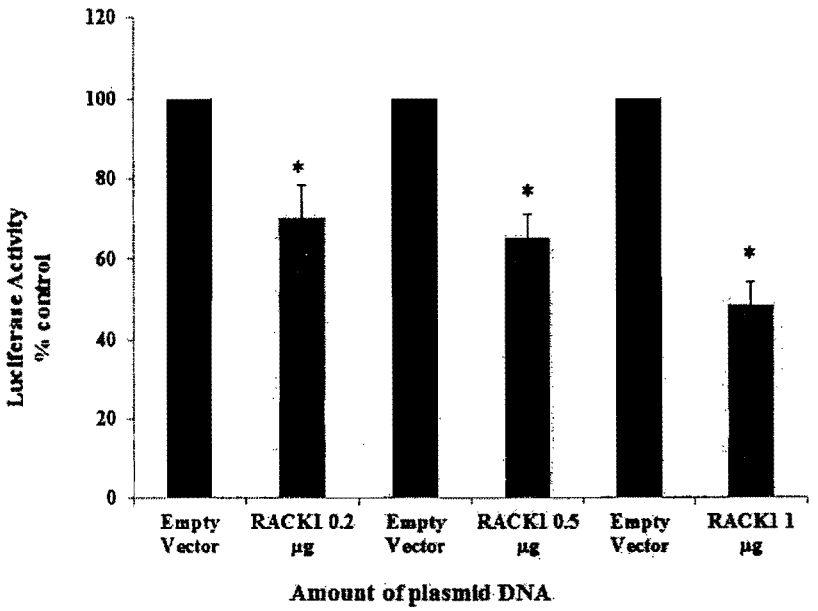


Figure 13: RACK 1 regulates the Mu opioid (MOR) promoter activity

Results from Luciferase reporter gene assay, NMB cells were transiently transfected either with pcDNA3-RACK1 (textured bar) and pcDNA3.1 vector (Black bars as control) along with p357 plasmid containing the human active MOR promoter in the luciferase reporter pGL3-basic vector. After forty-eight hours of transfection, the luciferase activity of the cell extracts was quantified using a luminometer and the activity of control (empty vector) without the RACK1 plasmid was compared to the three different concentrations of RACK1 plasmid co-transfected with plasmid containing hMOR promoter. Promoter activity of each construct was expressed as n-fold activation of pGL3-basic plasmid activity. Histograms represent mean values of n-fold activation. In cells with RACK1 overexpression the hMOR promoter activity was reduced in a dose dependent manner from 20% with lowest concentration (0.2 µg) to 50% with highest concentration (1µg). The luciferase data was normalized using protein concentration present in each sample and the histograms represent mean ± SE from six different experiments. Only the plus error bars are shown in the figure. "*" indicates $p < 0.01$ (Student's paired t-test).

Effect of RACK1 siRNA knockdown on RACK1 expression levels

To further confirm the regulatory effect shown in Fig. 13, RACK1-PCBP1 interaction has shown to regulate hMOR gene expression, RACK1 siRNA knockdown experiment was performed. Several concentrations (50, 100 and 200 nM) of RACK1 siRNA were transfected into the NMB cells while mock transfected cells were used as control. Forty eight hours after transfection, total RNA was isolated and RT-PCR was performed using primers for RACK1. Human β-actin primers were included in every PCR reaction as an internal control. The use of increasing amount of RACK1 siRNA has shown to decrease RACK1 mRNA levels Fig. 14A. The amount of endogenous RACK1 mRNA from the control cells was defined as 100% .The quantified histograms Fig. 14B, have shown a significant decrease in RACK1 mRNA levels 80% with lowest concentration (50 nM) to 50% with highest concentration (200 nM) of RACK1 siRNA. Combined, these results have demonstrated that RACK1 siRNA successfully knocked down the RACK1 mRNA levels in the NMB cells.

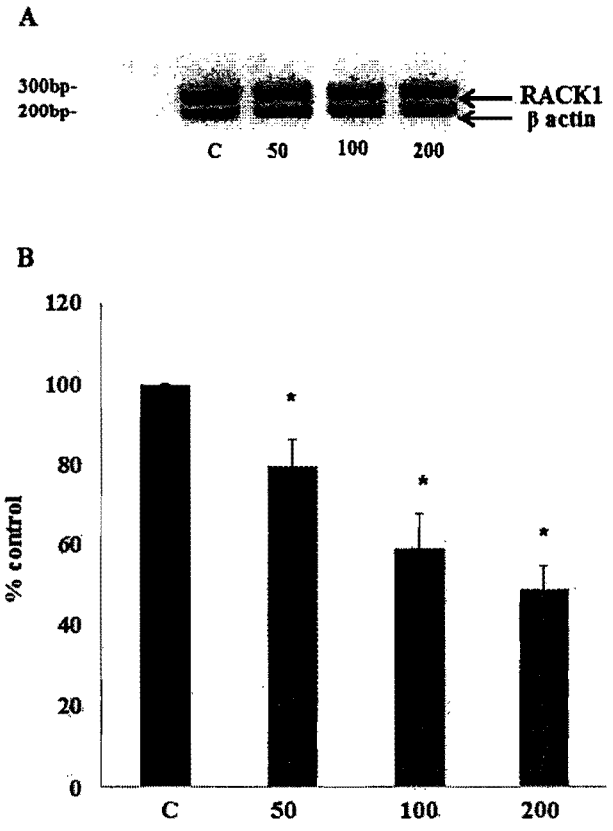


Figure 14: Knockdown of RACK1 mRNA levels using RACK1 siRNA

(A) Results from RT-PCR showing RACK1 gene expression levels in NMB cells transfected with three different concentrations of RACK1 siRNA. The mock transfected cells were used as control and human β-actin specific primers were included in every PCR reaction (added at cycle 12) as internal control for normalization. PCR products were analyzed on a 2% agarose gel containing 0.5 μg/ml of ethidium bromide and then quantified. (B) The normalized mRNA levels from control were defined as 100% and the quantified data has shown to decrease the RACK1 mRNA levels in cells transfected with RACK1 siRNA when compared to control. Shown here mRNA levels are presented as mean ± SE from six different experiments. Only the plus error bars are shown in the figure. "*" indicates $p < 0.01$ (Student's paired t-test).

Effect of RACK siRNA on hMOR mRNA levels

Since RACK1 siRNA has shown to successfully knockdown RACK1 mRNA levels (Fig. 14), effect of the RACK1 siRNA on the hMOR gene expression was further studied. Three different concentrations of RACK1 siRNA (50, 100, 200 nM) were used for transfections and forty-eight hours after the transfection and RT-PCR using primers specific for hMOR gene was performed. As shown in Fig. 15A, significant increase in the endogenous hMOR mRNA levels was observed in a dose dependent manner with increasing amounts of RACK1 siRNA. The quantified data of hMOR mRNA levels in the form of histograms are shown in Fig. 15B. In each RT-PCR reaction β-actin was used as an internal standard for data normalization and the amount of hMOR mRNA from the control cells was defined as 100%. Collectively, data from luciferase assay (Fig. 13) and RACK1 siRNA experiments (Fig. 15) have shown that RACK1 negatively regulates hMOR gene expression.

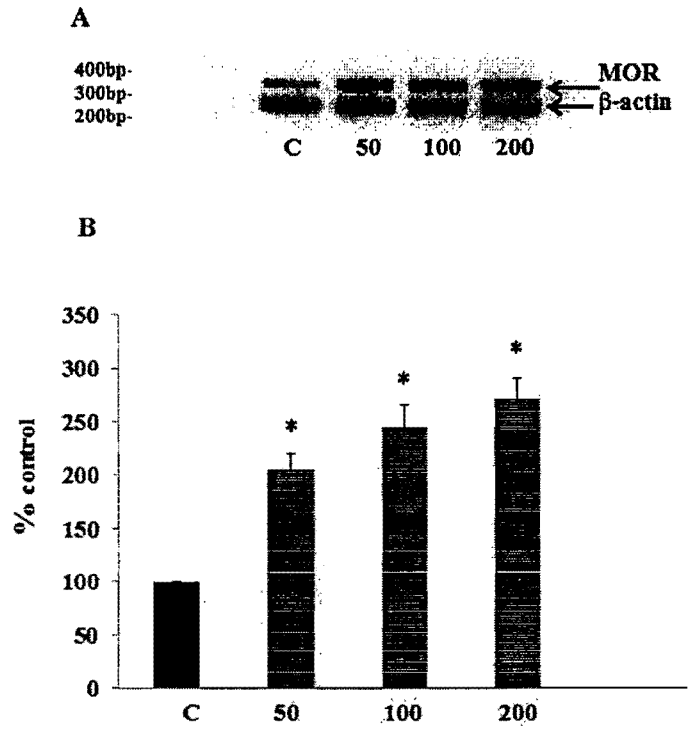


Figure 15: Effect of RACK1 siRNA on hMOR mRNA levels

(A) Results of RT-PCR showing hMOR gene expression levels in NMB cells transfected with three different concentrations of RACK1 siRNA. The mock transfected cells were used as control and human β-actin specific primers were included in every PCR reaction (added at cycle 12) as internal control for normalization. PCR products were analyzed on a 2 % agarose gel containing 0.5 μg/ml of ethidium bromide and then quantified. (B) The normalized mRNA levels from control were defined as 100% and the quantified data has shown increase in the hMOR mRNA levels in cells transfected with RACK1 siRNA when compared to control. Shown here mRNA levels are presented as mean ± SE from six different experiments. Only the plus error bars are shown in the figure. "*" indicates $p < 0.01$ (Student's paired t-test).

Effect of RACK1 knockdown on RACK1 protein level

To further investigate whether changes in RACK1 mRNA also reduces RACK1 protein levels, NMB cells were transfected with 200 nM of RACK1 siRNA and Western blot analysis of these transfected sample was performed. As there was no significant change in RACK1 protein levels forty-eight hours after transfection (Fig. 16A), the changes in the RACK1 mRNA and protein levels were studied after seventy-two hours. As shown in Fig. 16B, RACK1 mRNA levels remained low as compared to the control. After seventy-two hours of transfection Western blot analysis was performed as shown in Fig. 16C, the endogenous RACK1 protein level were significantly reduced. Anti-β-actin antibody was used as an internal standard for normalization. The quantified data of the Western blot analysis Fig. 16D, has shown decrease in the RACK1 protein levels (approximately 50%) when compared to the control (defined as 100%). These results demonstrated that the RACK1 protein levels were significantly reduced after seventy-two hours of transfection while there was no significant decrease in the RACK1 protein levels after forty-eight hours of transfection with RACK1 siRNA.

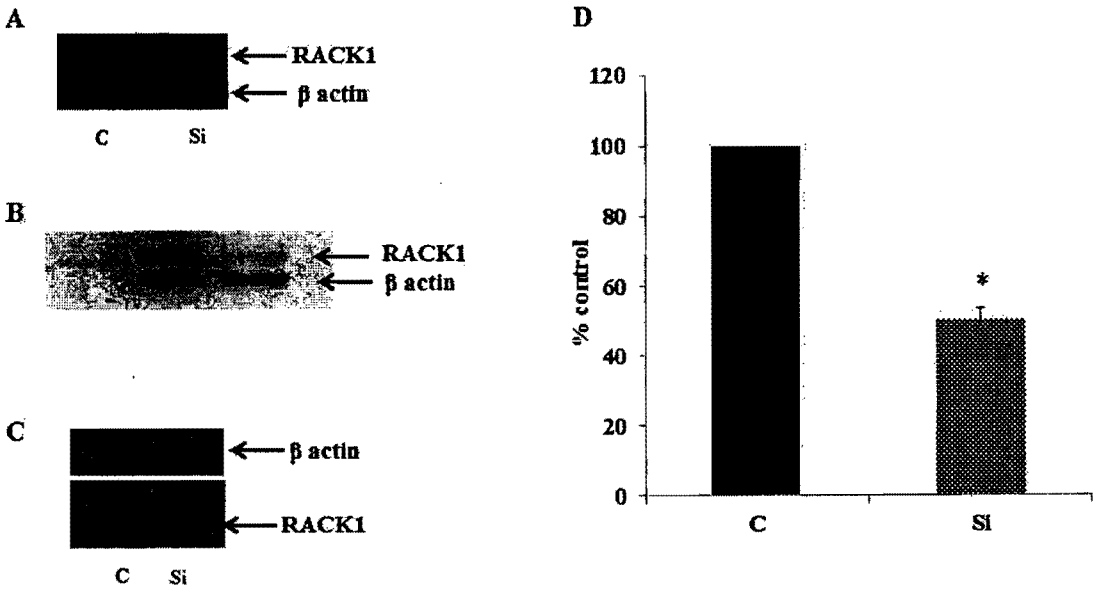


Figure 16: Effect of RACK1 siRNA on RACK1 protein level

(A) Results from Western blot analysis of RACK1 protein levels after forty eight hours post transfection using anti-RACK1 antibody along with human β-actin antibody as an internal standard. There is no significant change in amount of RACK1 protein in siRNA transfected sample when compared to the control. (B) Results from RT-PCR of RACK1 mRNA expression after seventy-two hours of transfection of NMB cells with 200 nM RACK1 siRNA and mock transfected cells as control. There was significant decrease in the RACK1 mRNA levels. (C) Results from Western blot analysis of RACK1 protein levels after seventy-two hours of transfection using anti-RACK1 antibody along with human β-actin antibody as an internal standard for normalization purpose. (D) The normalized protein levels of RACK1 protein from the control were defined as 100 %. There is decrease in RACK1 protein levels approximately 50% when compared to control shown by the quantified histograms. The data shown in quantified histograms are presented as mean ± SE from six different experiments. Only the plus error bars are shown in the figure. "*" indicates $p < 0.01$ (Student's paired t-test).

Effect of RACK1 siRNA on PCBP1

Interaction between RACK1 and PCBP1 has shown to have a functional effect on hMOR gene expression (Figs. 13 and 15). PCBP1 has also been known to regulate hMOR gene expression (Cook et al., 2010). Thus, the effect of RACK1 siRNA on PCBPl in NMB cells was further investigated. After seventy-two hours of transfection RT-PCR using primers specific to PCBP1 was performed. There was no significant change in levels of PCBP1 mRNA Fig. 17A, when compared to the control. β-actin was used as an internal standard and the quantified PCBP1 mRNA levels are shown in Fig. 17B. The normalized PCBP1 mRNA level from the control was defined as 100%. In cells transfected with RACK1 siRNA no significant difference in PCBP1 mRNA levels was observed.

PCBP1 protein levels were also examined by Western blot analysis using anti-PCBP antibody and anti- β-tubulin antibody was used as internal standard for data normalization. As shown in Fig. 17C and 17D, there was no significant decrease in the PCBP1 protein levels. Combined, results from the RT-PCR and the Western blot analysis show that there is no significant effect on endogenous PCBP1 mRNA and protein levels in cells transfected with RACK1 siRNA.

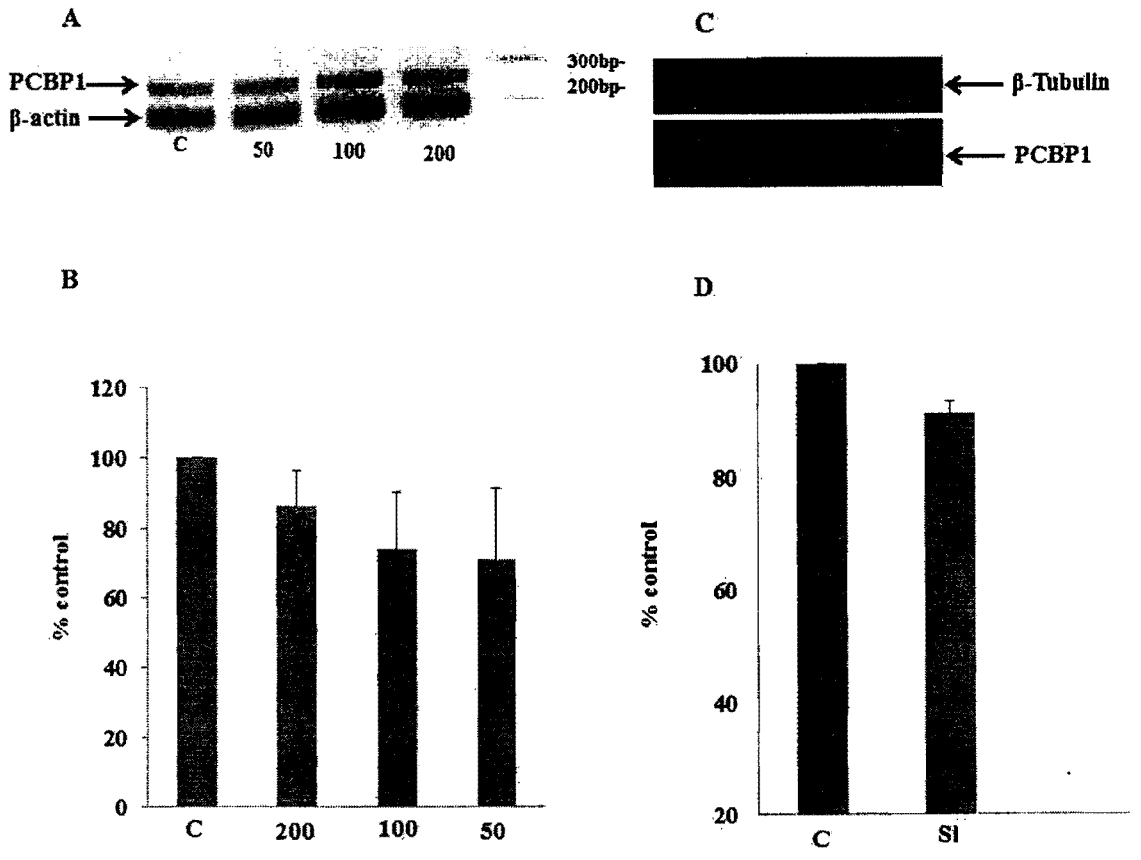


Figure 17: Effect of RACK1 siRNA on PCBP1 mRNA and protein levels.

(A) Results from RT-PCR analysis of PCBP1 mRNA levels seventy-two hours after transfection with 200 nM RACK1 siRNA and mock transfected cells as control. PCR products were analyzed on a 2% agarose gel electrophoresis. (B) Quantified histogram of the normalized PCBP1 mRNA level from control was defined as 100 % and has shown no significant difference in PCBP1 mRNA levels between control and RACK1 siRNA transfected cells. (C) Results from Western blot analysis of PCBP1 protein levels using anti-PCBP1 antibody. Human β-Tubulin antibody was used as an internal standard for normalization purpose. (D) Quantified histogram of PCBP1 protein levels has shown no significant difference in the PCBP1 protein levels in cells transfected with RACK siRNA when compared to control. The histograms shown here are presented as mean ± SE from five different experiments. Only the plus error bars are shown in the figure.

Effect of RACK1 siRNA on the hMOR protein levels

To further investigate whether changes in hMOR mRNA levels also had an effect on the hMOR protein levels, radiolabeled receptor binding experiments were performed. In cells transfected with RACK1 siRNA an increase hMOR mRNA levels was observed Fig. 13A and 13B. Thus, the effect of RACK1 siRNA on hMOR mRNA and protein levels seventy-two hours after transfection was further investigated. After seventy two hours of transfection with RACK1 siRNA, the mRNA levels of hMOR were still elevated when compared to control (Fig. 18A) thus, the level of hMOR protein was further examined by radiolabeled receptor binding assay. The hMOR protein is a receptor which can bind to opioid ligand such as diprenorphine. Using radioisotope labeled ^{3}H-diprenorphine, receptor binding assay was performed with 1 μM CTAP as the competitive ligand. As NMB cells express all three opioid receptors, μ, δ, and κ (Baumhaker et al., 1994), the two sub-types (κ and δ) receptors, were blocked using receptor subtype selective ligands DADLE (δ) and U50488 (κ) at a concentration of 1 μM. Specific binding activity was calculated as count per minute (CPM)/mg of protein shown in Fig. 18B, where a significant increase in hMOR receptor binding in cells transfected with RACK1 siRNA was observed when compared to control cells. Percent binding of receptor was also calculated as shown Fig. 18C, where specific binding from the control was defined as 100 % and the binding data was normalized for equivalent amount of protein present in control and siRNA transfected cells. Significant increase in the percent binding in cells transfected with RACK1 siRNA was observed. As shown from the Fig. 18B and 18C, siRNA knockdown of RACK1 has resulted in an increase of hMOR mRNA level and also resulted in increase of hMOR protein level at seventy two hours post transfection. In summary, RACK1 knockdown decreases the endogenous RACK1 protein level, which results in increase in the endogenous hMOR mRNA and hMOR

protein levels in the NMB cells. This data supports the finding of the luciferase reporter gene assay (Fig. 13), that RACK1 regulates the MOR expression levels by interacting with PCBP1.

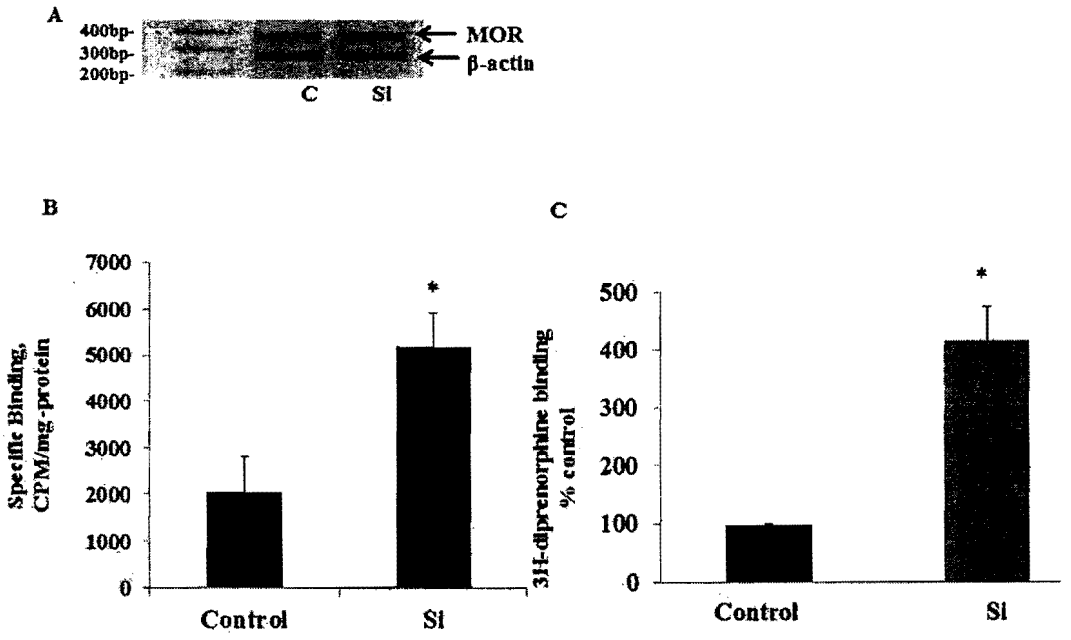


Figure 18: Effect of RACK1 knockdown on MOR protein level

(A) Results from RT-PCR analysis showing hMOR mRNA levels seventy-two hours after transfection with RACK1 siRNA, human β-actin specific primers were included in every PCR reaction (added at cycle 12) as an internal standard. PCR products were separated and analyzed on a 2% agarose gel. Increase in hMOR mRNA levels in samples with RACK1 knockdown when compared to the control mock transfected cells was seen (B) The hMOR protein levels were determined using competitive receptor binding experiments using radio labeled ^{3}H-diprenorphine and 1μM CTAP as the competitive ligand. To block the other two (κ and δ) subtype receptor specific ligands DADLE (δ) and U50488 (κ) were used at concentration of 1 μM. Specific binding activity defined as CPM/mg protein was calculated and shown in form of histograms. The data from specific binding activity as CPM/mg protein has shown increase the

binding in cells transfected with RACK1 siRNA when compared to control. (C) Percent specific binding was also calculated where binding from the control was defined as 100% and the binding data was normalized for equivalent amount of protein present in control and siRNA transfected cells. Quantified histograms of receptor binding levels are presented as mean ± SE from three independent experiments. Only the plus error bars are shown in the figure. "*" indicates $p < 0.01$ (Student's paired t-test)

Discussion

In this study, we report the identification of RACK1 as PCBP1 interacting protein and have presented evidence for the physical interaction between RACK1 and PCBP1. The physical interaction between RACK1 and PCBP1 was studied by two independent experiments; the bacteria two hybrid screening and co-immunoprecipitation assay of endogenous proteins.

PCBP1 is known to act as a transcriptional regulator of hMOR gene (Ko and Loh, 2005, Malik et al., 2006, Cook et al., 2010). Therefore, it was hypothesized that the physical interaction between PCBP1 and RACK1 can modulate the hMOR gene expression. RACK1 functional effect of RACK1 on the hMOR gene expression is supported by the findings that RACK1 overexpression decreases the hMOR promoter activity (Fig. 13). RACK1 siRNA knockdown experiments, have shown to decrease RACK1 mRNA levels which causes increase in the hMOR mRNA and hMOR mRNA levels (Figs. 14 and 15). The changes in the hMOR and RACK1 mRNA levels were evident faster within forty-eight hours of transfection when compared to changes in the RACK1 protein levels (Fig. 16), which required seventy-two hours after transfection.

RACK1 was originally cloned from both a chicken liver cDNA library and a human B-lymphoblastic cell line and is referred to as C12.3 or H12.3, respectively (Guillemot et al., 1989). The name RACK1 was adopted by a group describing its ability to bind activated Protein kinase C (PKC) (Mochly-Rosen and Gordon, 1998). RACK1 is a 36 kDa cytosolic protein containing Trp-Asp 40 (WD40) repeats that are responsible for mediating its protein–protein interaction properties (Imai et al., 2009). Using the bacteria two hybrid system, the interaction site of RACK1 and PCBP1 was mapped. The deletion studies have confirmed that the binding of RACK1 and PCBP1 requires a critical region in the N-terminus within the WD 7 repeat of

RACK1 (Figs. 9 and 12), for WD7 alone has shown to possess the ability to interact with PCBP1. The other six WD domains have shown to possess minor to no ability to interact with RACK1. These results were also supported by the serial WD deletion experiments (Fig. 12), where WD7 alone has shown to possess strongest ability to interact with PCBP1. The differences in the interaction capacity of the WD domains of RACK1 could be due to the differences in the individual WD motifs, although each of the WD domains has a similar bladed structure.

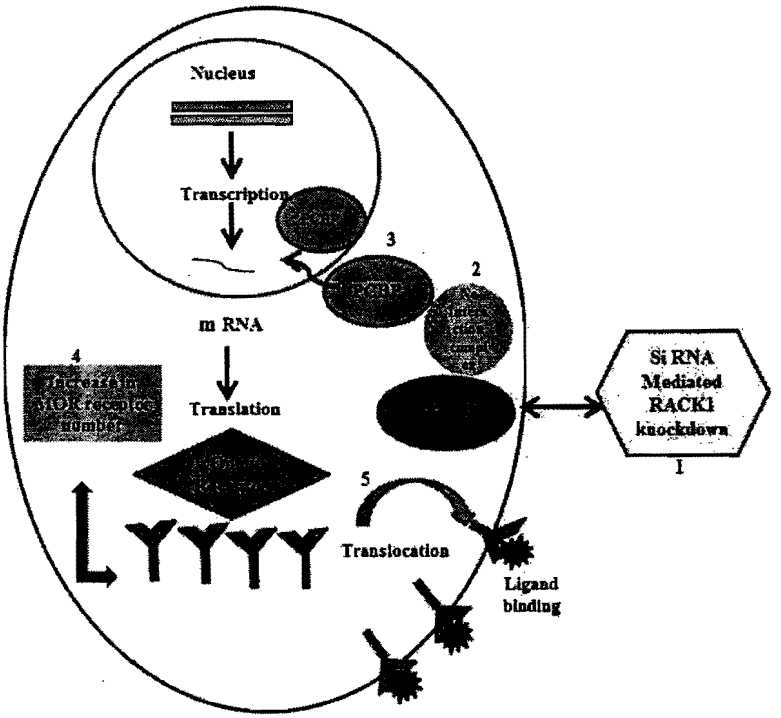
The presence of WD motifs makes RACK 1 share structural properties with the G protein β subunit while the WD repeats of RACK1 form a seven-bladed propeller structure where each blade is made up of β sheets (Buensuceso et al., 2001, Sondek and Siderovski, 2001). In a diverse range of species from plants and arthropods like *Drosophila melanogaster* to nematodes like *Caenorhabditis elegans*, the WD repeat sequence is well conserved (Nakashima et al., 2008, Ai et al., 2009). Despite their highly conserved structural motif, WD40 proteins play very diverse functions due to the ability of these proteins to coordinate the binding of a variety of proteins through the individual blades (Chen et al., 2004).

In most of the WD proteins, the WD repeats are connected by loops of variable size which project above and below of the propeller structure, the β sheets are more conserved both in the size and properties as compared to the loops. The difference in the structural properties of the WD repeats are responsible for providing salient features to each member of the WD family (Garcia-Higuera et al., 1998). In RACK1, the distinct sheets are splayed outward, giving it a conical shape with the three interaction sites top, bottom and the circumference. The main chain hydrogen bond formed between the β strands in the propeller itself supports the interactions with the other partners. An interacting protein partner would interact at the sides of the propeller in addition to interactions with other protein partner below the propeller or along its circumference

(Sklan et al., 2006). The seventh WD repeat of RACK1 has been known to interact with several proteins examples of these protein partners are integrin β-sub unit, Protein phosphatase 2 (PP2A) and Factor associated with neural sphingomyelinase activation (FAN) (Liliental and Chang, 1998, Tcherkasowa et al., 2002, Kiely et al., 2008). RACK1 interacts with the androgen receptor and promotes cross talk via PKC signaling through the WD 6 domain and domains WD1-4 are responsible for RACK1-insulin receptor/ IGF1R interaction (Rigas et al., 2003, Zhang et al., 2006). Thus, taken together RACK1 is known to interact with several proteins via one or several of its characteristic WD domains.

The RACK1 gene is universally expressed in the tissues of mammals and humans including brain, liver, and spleen, suggesting that it has an important functional role (Guillemot et al., 1989, Chou et al., 1999). There are several studies reporting various functional roles of RACK1. RACK1 appears to serve as a "scaffold" or "anchor" protein for PKC isoforms, in this mode of function it serves neither as a substrate nor an inhibitor, but enables translocation and stabilization of PKC isoforms (Mochly-Rosen and Gordon, 1998). In aging rat brains studies have demonstrated that reduction of RACK1 levels correlates to malfunctioning of PKC translocation (Battaini et al., 1997). The disruption of PKC-RACK1 interactions have shown to impair insulin-induced kinase translocation and *Xenopus* oocyte maturation and in cardiomyocytes the calcium channels are regulated by RACK1 (Ron et al., 1995, Zhang et al., 2006). Studies have suggested that RACK1-Src kinase interaction causes a decrease in the level of Src activity and cell growth rates in NIH 3T3 cells that stably over-express RACK1 (Chang et al., 1998). The data presented here along with previous published work suggests that RACK1 plays opposing roles depending on the protein with which it interacts.

In this study, we have shown that RACK1 localizes mainly in the cytosol when compared to the nucleus (Fig. 4), suggesting its potential functional role in the nucleus. To shed insight on the functional effect of the interaction between RACK1 and PCBP1, spatial significance of this interaction was studied. Results indicate that the interaction between RACK1 and PCBP1 occurs outside the nucleus in the cytosol. Post translational modification of PCBP1 in form phosphorylation has shown to affect nuclear translocation ability (Meng et al., 2007) and may affect the interaction between RACK1 and PCBP1 within the nucleus.



Using human neuronal NMB cell system how RACK1 and PCBP1 interact and modulate the hMOR gene expression is proposed in the above schematic representation. SiRNA mediated RACK1 knockdown decreases expression of RACK1 protein (1), as a result of this RACK1 knockdown, PCBP1 and RACK1 do not form an interaction complex (2), PCBP1 now trans locates to the nucleus (3), where it is known to act as a transcription regulator of hMOR gene by increasing the MOR transcription (Ko and Loh, 2005, Malik et al., 2006, Cook et al., 2010) and increases the expression hMOR protein level via post-transcriptional events (4) as well as translational and post-translational events (5). Conversely, when RACK1 is overexpressed via transfection, RACK1 interacts with PCBP1 generating more PCBP1-RACK1 complexes thus limiting the availability of free PCBP1 to regulate the hMOR expression by translocating in to the nucleus.

Conclusion

In conclusion, this study has identified RACK1 as the PCBP1 interacting protein and can regulate the hMOR gene expression using a human neuronal cell system. This new functional role of RACK1 and its interaction with PCBP1 can provide further understanding about hMOR regulation.

References

Ai E, Poole DS, Skop AR (2009). RACK-1 directs dynactin-dependent RAB-11 endosomal recycling during Mitosis in *Caenorhabditis elegans*. Molecular Biology of the Cell 20:1629-1638.

Bare LA, Mansson E, Yang D (1994). Expression of two variants of the human mu opioid receptor mRNA in SK-N-SH cells and human brain. Federation of European Biochemical Societies Letters 354:213-216.

Battaini F, Pascale A, Paoletti R, Govoni S (1997). The role of anchoring protein rack1 in pkc activation in the ageing rat brain. Trends in Neuroscience 20:410-415.

Baumhaker Y, Ben-Dor T, Bar-Hamburger R, Sarne Y (1994). Characterization of a triple opioid system in the human neuroblastoma NMB cell line. Brain Research 665:94-100.

Blyn LB, Swiderek KM, Richards O, Stahl DC, Semler BL, Ehrenfel (1996). Poly (rC) binding protein 2 binds to stem-loop IV of the poliovirus RNA 59 noncoding region: identification by automated liquid chromatography-tandem mass spectrometry. Proceedings of the National Academy of Sciences (USA) 93:11115–11120.

Buensuceso CS, Woodside D, Huff JL, Plopper GE, O'Toole TE (2001). The WD protein Rack1 mediates protein kinase C and integrin-dependent cell migration. Journal of Cell Science 14:1691-1698.

Chang BY, Conroy KB, Machleder EM, Cartwright CA (1998). RACK1, a receptor for activated C kinase and a homolog of the beta subunit of G proteins, inhibits activity of src tyrosine kinases and growth of NIH 3T3 cells. Molecular Cell Biology 18:3245-3256.

Chen S, Spiegelberg BD, Lin F, Dell EJ, Hamm HE (2004). Interaction of Gβγ with RACK1 and other WD40 repeat proteins. Journal of Molecular and Cellular Cardiology 37:399-406.

Chen Y, Mestek A, Liu J, Hurley JA, Yu L (1993). Molecular cloning and functional expression of a mu-opioid receptor from the rat brain. Molecular Pharmacology 44:8-12.

Choi HS, Hwang CK, Song KY, Law P-Y, Wei L-N, Loh HH (2009). Poly (C) binding proteins as transcriptional regulators of gene expression. Biochemical and Biophysical Research Communications 380:431-436.

Chou YC, Chou CC, Chen YK, Tsai S, Hsieh FM, Liu HJ, Hseu TH (1999). Structure and genomic organization of porcine RACK1 gene. Biochimica et Biophysica Acta 23:2-3.

Cloke B, Shah K, Kaneda H, Lavery S, Trew G, Fusi L, Higham J, Dina RE, Ghaem-Maghami S, Ellis P, Brosens JJ, Christian M (2010). The Poly (C) binding protein 1 regulates expression of the androgen receptor. Journal of Endocrinology 151:3954-3964.

Cook RJ, Karch C, Nahar P, Rivera A, Ko JL (2010). Effects of desferoxamine-induced hypoxia on neuronal human mu-opioid receptor gene expression. Biochemical and Biophysical Research Communications 398:56-61.

Dawid IB, Breen JJ, Toyama R (1998). LIM domains: multiple roles as adapters and functional modifiers in protein interactions. Trends in Genetics 14:156-162.

Dietis N, Rowbotham DJ, Lambert DG (2011). Opioid receptor subtypes: fact or artifact? British Journal of Anaesthesia 107:8-18.

Ebrahimi S,Gregory SL (2011). Dissecting protein interactions during cytokinesis. Journal of Communicative and Intergrative Biology 4:243-244.

Evans, C. J., Keith, D. E., Jr., Morrison, H., Magendzo, K. and Edwards, R. H. (1992). Cloning of a delta opioid receptor by functional expression. Science 258:1952-1955.

Farhang-Fallah J, Randhawa VK, Nimnual A, Klip A, Bar-Sagi D, Rozakis-Adcock M (2002). The Pleckstrin homology (PH) domain interacting protein couples the insulin receptor substrate 1 PH domain to insulin signaling pathways leading to mitogenesis and GLUT4 translocation. Molecular Cell Biology 22:7325-7336.

Ferguson SS, Barak LS, Zhang J, Caron MG (1996). G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins. Canadian Journal of Physiology and Pharmacology 74:1095-1110.

Funke B, Zuleger B, Benavente R, Schuster T, Goller M, Stevenin J, Horak I (1996). The mouse poly (C) binding protein exists in multiple isoforms and interacts with several RNA-binding proteins. Nucleic Acids Research 24:3821-3828.

Gamarnik AV, Andino R (1997). Two functional complexes formed by KH domain containing proteins with the 59 noncoding region of poliovirus RNA. The Journal of Ribo Nucleic Acid 3:882-892.

Gaillard C, Cabannes E, Strauss F (1994). Identity of the RNA-binding protein K of hnRNP particles with protein H16, a sequence specific single strand DNA-binding protein. Nucleic Acids Research 22:4183-4186.

Gharagozlou P, Demirci H, David Clark J, Lameh J (2003). Activity of opioid ligands in cells expressing cloned mu opioid receptors. Biomed Central Pharmacology 3:1471-2210.

Guillemot F, Billault A, Auffray C (1989). Physical linkage of a guanine nucleotide-binding protein-related gene to the chicken major histocompatibility complex. Proceedings of the National Academy of Sciences (USA) 86:4594-4598.

Hoffman DW, Query CC, Golden BL, White SW, Keene JD (1991). RNA-binding domain of the A protein component of the U1 small nuclear ribonucleoprotein analyzed by NMR spectroscopy is structurally similar to ribosomal proteins. Proceedings of the National Academy of Sciences (USA) 88:2495-2499.

Imai S-i, Yasuda S, Kai M, Kanoh H, Sakane F (2009). Diacylglycerol kinase delta associates with receptor for activated C kinase 1, RACK1. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1791:246-253.

Kanjhan R (1995). Opioids and pain. Clinical and Experimental Pharmacology and Physiology 22:397-403.

Kieffer BL, Befort K, Gaveriaux-Ruff C, Hirth CG (1992). The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. Proceedings of the National Academy of Sciences (USA) 89:12048-12052.

Kieffer BL, Gaveriaux-Ruff C (2002). Exploring the opioid system by gene knockout. Progress in Neurobiology 66:285-306.

Kiledjian M, DeMaria CT, Brewer G, Novick K (1997). Identification of AUF1 (heterogeneous nuclear ribonucleoprotein D) as a component of the alpha-globin mRNA stability complex. Molecular and Cellular Biology 17:4870-4876.

Kim S-S, Pandey KK, Choi HS, Kim S-Y, Law P-Y, Wei L-N, Loh HH (2005). Poly (C) binding protein family is a transcription factor in mu opioid receptor gene expression. Molecular Pharmacology 68:729-736.

Knapp RJ, Malatynska E, Collins N, Fang L, Wang JY, Hruby VJ, Roeske WR, Yamamura HI (1995). Molecular biology and pharmacology of cloned opioid receptors. The Federation of American Societies for Experimental Biology 9:516-525.

Ko JL, Minnerath SR, Loh HH (1997). Dual promoters of mouse mu-opioid receptor gene1. Biochemical and Biophysical Research Communications 234:351-357.

Ko JL, Liu H-C, Loh HH (2003). Role of an AP-2-like element in transcriptional regulation of mouse mu-opioid receptor gene. Molecular Brain Research 112:153-162.

Ko JL, Loh HH (2005). Poly (C) binding protein, a single-stranded DNA binding protein, regulates mouse mu-opioid receptor gene expression. Journal of Neurochemistry 93:749-761.

Krecic AM, Swanson MS (1999). hnRNP complexes: composition, structure, and function. Currents Opinions in Cell Biology 11, 363–371.

Lin Y-C, Flock KE, Cook RJ, Hunkele AJ, Loh HH, Ko JL (2008). Effects of trichostatin A on neuronal mu-opioid receptor gene expression. Brain Research 1246:1-10.

Lynch M, Chen L, Ravitz MJ, Mehtani S, Korenblat K, Pazin MJ, Schmidt EV (2005). hnRNP K binds a core polypyrimidine element in the eukaryotic translation initiation factor 4E (eIF4E) promoter, and its regulation of eIF4E contributes to neoplastic transformation. Molecular and Cellular Biology 25:6436-6453.

Makeyev AV, Liebhaber SA. 2002. The poly (C) binding proteins: A multiplicity of functions and a search for mechanisms. RNA , Journal of the RNA Society 8: 265–278.

Malik AK, Flock KE, Godavarthi CL, Loh HH, Ko JL (2006). Molecular basis underlying the Poly (C) binding protein 1 as a regulator of the proximal promoter of mouse mu-opioid receptor gene. Brain Research 1112:33-45.

McCahill A, Warwicker J, Bolger GB, Houslay MD, Yarwood SJ (2002). The RACK1 scaffold protein: a dynamic cog in cell response mechanisms. Molecular Pharmacology 62:1261-1273.

Michelotti GA, Michelotti EF, Pullner A, Duncan RC, Eick D, Levens D (1996). Multiple single-stranded cis elements are associated with activated chromatin of the human *c-myc* gene *in vivo*. Molecular and Cellular Biology 16:2656-2669.

Min BH, Augustin LB, Felsheim RF, Fuchs JA, Loh HH (1994). Genomic structure analysis of promoter sequence of a mouse mu-opioid receptor gene. Proceedings of the National Academy of Sciences (USA) 91:9081-9085.

Mochly-Rosen D, Gordon AS (1998). Anchoring proteins for protein kinase C: a means for isozyme selectivity. The Federation of American Societies for Experimental Biology 12:35-42.

Morris AJ, Martin SS, Haruta T, Nelson JG, Vollenweider P, Gustafson TA, Mueckler M, Rose DW, Olefsky JM (1996). Evidence for an insulin receptor substrate 1 independent insulin signaling pathway that mediates insulin-responsive glucose transporter (GLUT4) translocation. Proceedings of National Academy of Sciences (USA) 16:8401-8406.

Musco G, Stier G, Joseph C, Morelli MAC, Nilges M, Gibson TJ, Pastore A (1996). Three-dimensional structure and stability of the KH domain: Molecular insights into the fragile X syndrome. Cell 85:237-245.

Nakashima A, Chen L, Thao NP, Fujiwara M, Wong HL, Kuwano M, Umemura K, Shirasu K, Kawasaki T, Shimamoto K (2008). RACK1 functions in rice innate immunity by interacting with the Rac1 immune complex. Plant Cell 20:2265-2279.

Narita M, Mizoguchi H, Narita M, Sora I, Uhl GR, Tseng LF (1999). Absence of G-protein activation by μ-opioid receptor agonists in the spinal cord of μ-opioid receptor knockout mice. British Journal of Pharmacology 126:451-456.

Pasternak GW (2005). Molecular biology of opioid analgesia. Journal of Pain and Symptom Management 29:2-9.

Rigas AC, Ozanne DM, Neal DE, Robson CN (2003). The scaffolding protein RACK1 interacts with androgen receptor and promotes cross-talk through a protein kinase C signaling pathway. Journal of Biological Chemistry 278:46087-46093.

Rivera-Gines A, Cook RJ, Loh HH, Ko JL (2006). Interplay of Sps and poly (C) binding protein 1 on the mu-opioid receptor gene expression. Biochemical and Biophysical Research Communications 345:530-537.

Ron D, Luo J, Mochly-Rosen D (1995). C2 Region-derived peptides inhibit translocation and function of protein kinase C *in vivo*. Journal of Biological Chemistry 270:24180-24187.

Schullery DS, Ostrowski J, Denisenko ON, Stempka L, Shnyreva M, Suzuki H, Gschwendt M, Bomsztyk K (1999). Regulated interaction of protein kinase Cδ with the heterogeneous nuclear ribonucleoprotein K protein. Journal of Biological Chemistry 274:15101-15109.

Simonds WF (1988). The molecular basis of opioid receptor function. Endocrine Reviews 9:200-212.

Siomi H, Choi M, Siomi MC, Nussbaum RL, Dreyfuss G (1994). Essential role for KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. Cell 77:33-39.

Sklan EH, Podoly E, Soreq H (2006). RACK1 has the nerve to act: Structure meets function in the nervous system. Progress in Neurobiology 78:117-134.

Sondek J, Siderovski DP (2001). G γ-like (ggl) domains: new frontiers in g-protein signaling and β-propeller scaffolding. Biochemical Pharmacology 61:1329-1337.

Tomonaga T, Levens D (1995). Heterogeneous nuclear ribonucleoprotein K is a DNA-binding transactivator. Journal of Biological Chemistry 270:4875-4881.

Tomonaga T, Levens D (1996). Activating transcription from single stranded DNA. Proceedings of the National Academy of Sciences (USA) 93:5830–5835.

Valverde R, Edwards L, Regan L (2008). Structure and function of KH domains. Federation of European Biochemical Societies 275:2712-2726.

Wei L-N, Loh HH (2011). Transcriptional and epigenetic regulation of opioid receptor genes: present and future. Annual Reviews of Pharmacology and Toxicology 51:75-97.

Yang YYL, Yin GL, Darnell RB (1998). The neuronal RNA-binding protein Nova-2 is implicated as the autoantigen targeted in POMA patients with dementia. Proceedings of the National Academy of Sciences (USA) 95:13254-13259.

Yeagle PL, Albert AD (2007). G protein-coupled receptor structure. Biochimica et Biophysica Acta (BBA) - Biomembranes 1768:808-824.

Zhang T, Huang X-H, Dong L, Hu D, Ge C, Zhan Y-Q, Xu W-X, Yu M, Li W, Wang X, Tang L, Li C-Y, Yang X-M (2010). PCBP-1 regulates alternative splicing of the CD44 gene and inhibits invasion in human hepatoma cell line HepG2 cells. Molecular Cancer 9:1476-1498.

Zhang W, Zong CS, Hermanto U, Lopez-Bergami P, Ronai Ze, Wang L-H (2006). RACK1 recruits STAT3 specifically to insulin and insulin-like growth factor 1 receptors for activation, which is important for regulating anchorage-independent growth. Molecular Cell Biology 26:413-424.