Identification of RanBP 9/10 as Interacting Partners for Protein Kinase Cγ/δ and the D₁ Dopamine Receptor: regulation of PKC-mediated receptor phosphorylation

Elizabeth B. Rex, Michele L. Rankin, Yu Yang, Quansheng Lu, Charles R. Gerfen, Pedro A. Jose, and David R. Sibley

Molecular Neuropharmacology Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892-9405 (EBR, MLR, DRS)

Center for Molecular Physiology Research, Children’s Research Institute, Children’s National Medical Center, Washington, DC 20010 (YY, QL, PAJ)

Laboratory of System Neuroscience, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20892
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Address correspondence to: Dr. David R. Sibley
Molecular Neuropharmacology Section
NINDS/NIH
5625 Fishers Lane, Room 4S-04, MSC 9405
Bethesda, MD 20892-9405

Tel.: 301-496-9316
Fax: 301-480-3726
Email: sibley@helix.nih.gov.

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Abbreviations: GPCR, G protein-coupled receptor; PKC, protein kinase C; cAMP, adenosine 3’,5’-cyclic monophosphate; DA, dopamine; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DMEM, Dulbecco’s modified essential medium; FCS, fetal calf serum; HBS, HEPES buffered saline; EBSS, Earle’s Balanced Salt Solution; PVDF, polyvinylidene difluoride; TBST, Tris-buffered saline containing 0.05% Tween 20; EtOH, ethanol; mGluR, metabotropic glutamate receptor; MOR, mu-opioid receptor.
ABSTRACT

We previously reported that ethanol treatment regulates D1 receptor phosphorylation and signaling in a PKCδ and PKCγ-dependent fashion by a mechanism that may involve PKC isozyme-specific interacting proteins. Using a PKC isozyme-specific coimmunoprecipitation approach coupled to mass spectrometry, we report the identification of RanBP9 and RanBP10 as novel interacting proteins for both PKCγ and PKCδ. Both RanBP9 and RanBP10 were found to specifically co-immunoprecipitate with both PKCγ and PKCδ; however, this association did not appear to mediate the ethanol regulation of the PKCs. Interestingly, the D1 receptor was also found to specifically co-immunoprecipitate with RanBP9/10 from HEK293T cells and with endogenous RanBP9 from rat kidney. RanBP9 and RanBP10 were also found to co-localize at the cellular level with the D1 receptor in both kidney and brain tissue. Although over-expression of RanBP9 or RanBP10 in HEK293T cells did not appear to alter the kinase activities of either PKCδ or PKCγ, both RanBP proteins regulated D1 receptor phosphorylation and signaling, as well as expression in the case of RanBP9. Specifically, over-expression of either RanBP9 or RanBP10 enhanced basal D1 receptor phosphorylation, which was associated with attenuation of D1 receptor-stimulated cAMP accumulation. Moreover, treatment of cells with select PKC inhibitors blocked the RanBP9/10-dependent increase in basal receptor phosphorylation suggesting that phosphorylation of the receptor by PKC is regulated by RanBP9/10. These data support the idea that RanBP9 and RanBP10 may function as signaling integrators and dictate the efficient regulation of D1 receptor signaling by PKCδ and PKCγ.
INTRODUCTION

Dopamine (DA) is a key signaling molecule in the brain and periphery. The actions of this neurotransmitter are mediated by dopamine receptors, which are seven transmembrane spanning proteins belonging to the large family of G-protein coupled receptors. Dopamine receptors are divided into two major subfamilies, referred to as D1-like and D2-like, on the basis of their structure, pharmacology, and function (Missale et al., 1998). The D1-like receptors consist of the D1 and D5 subtypes, which couple to Gsα proteins to activate adenylyl cyclase and promote the accumulation of intracellular cAMP. In contrast, the D2-like receptors, which consist of the D2, D3 and D4 subtypes, couple to Gi/ο proteins, which tend to inhibit adenylyl cyclase and decrease intracellular cAMP levels.

The D1 receptor is abundantly expressed in the forebrain and it is not surprising that aberrant D1 receptor signaling has been linked to various neuropsychiatric disorders such as substance abuse, schizophrenia and Parkinson’s disease. For example, ethanol (EtOH) consumption is reduced in genetically modified mice that lack the D1 receptor, or wild type mice that are administered D1 selective antagonists, SCH23390 (El-Ghundi et al., 1998; Price and Middaugh, 2004). Conversely, treatment with D1 selective agonists facilitates EtOH related behaviors (D’Souza et al., 2003). Additionally, D1 selective agonists have been shown to ameliorate the cognitive deficits associated with schizophrenia and improve parkinsonian-like symptoms in animal models (Kebabian et al., 1992; McLean et al., 2009; Schneider et al., 1994). Importantly, understanding how D1 receptors are regulated may be useful for future therapeutic interventions.

Receptor phosphorylation is an important post-translational modification that regulates D1 receptor signaling. In particular, receptor phosphorylation generally contributes to desensitization - a process that renders the receptor less sensitive to subsequent agonist stimulation (Kohout and Lefkowitz, 2003). To date, three classes of protein kinases have been reported to phosphorylate the D1 receptor. These include the G protein-coupled receptor kinases (GRKs), cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). GRKs generally phosphorylate the D1 receptor under agonist-activated conditions, which results in receptor desensitization (Gainetdinov et al., 2004; Gardner et al., 2001; Rankin et al., 2006; Tiberi et al.,
1996). Similarly, PKA also regulates D₁ receptor signaling by modulating the rate of agonist-induced receptor desensitization and intracellular trafficking (Jiang and Sibley, 1999; Mason et al., 2002). In contrast to GRKs and PKA, very little is known about the regulation of the D₁ receptor by PKC phosphorylation. We recently determined that PKC constitutively phosphorylates the D₁ receptor and that this negatively regulates dopaminergic signaling (Rex et al., 2008). Moreover, we found that EtOH decreases constitutive PKC phosphorylation of the D₁ receptor with a concomitant potentiation of dopaminergic signaling (Rex et al., 2008). Importantly, EtOH was found to directly inhibit the enzymatic activities of PKCγ and PKCδ, but only when they were isolated from the plasma membrane fraction – an effect that was not observed for other PKC isozymes that were tested (Rex et al., 2008). The molecular mechanisms underlying the EtOH-mediated inhibition of membrane-associated PKCγ and PKCδ kinase activities, and how they target the D₁ receptor are at present unclear. One hypothesis for the membrane specificity of this effect is that PKC isozyme-specific interacting proteins exist in the plasma membrane and impart EtOH-sensitivity to PKCγ and PKCδ, or are the targets of EtOH themselves. Notably, a growing number of interacting proteins for PKCs have recently been identified (Piontek and Brandt, 2003; Rodriguez et al., 1999; Staudinger et al., 1997; Zemskov et al., 2003). Similarly, several D₁ receptor interacting proteins have also been reported that include various scaffolding/trafficking proteins (Bermak et al., 2001; Free et al., 2007; Hazelwood et al., 2008; Heydorn et al., 2004).

The aim of the current study was to use a proteomic-based approach to identify and characterize PKCγ/δ isozyme-specific interacting proteins that may be regulated by EtOH and associate with the D₁ dopamine receptor. We now report the identification of RanBP9 and RanBP10 as dual interacting proteins for both PKCγ/δ and the D₁ receptor. Although RanBP10 and RanBP9 do not appear to mediate EtOH-dependent inhibition of PKCδ or PKCγ, these interacting proteins were found to modulate D₁ receptor activity through increased receptor phosphorylation. Moreover, we found that the RanBP9/10-dependent increase in receptor phosphorylation is blocked by PKC inhibitor treatment, consistent with the idea that these scaffolding proteins may target PKCδ or PKCγ to the D₁ receptor.
MATERIALS AND METHODS

Materials. HEK293-tsa201 (HEK293T) cells were a gift from Dr. V. Ramakrishnan. HA-PKCδ and 3X-FLAG-PKCγ were gifts from Dr J.F. Mushinski (Mischak et al., 1993) and Dr. A. Newton, respectively. RanBP10-GFP and RanBPM-GFP were gifts from Dr. Guan Wu and Dr. Takeharu Nishimoto, respectively. PKCγ antibody was purchased from GeneTex, Inc. (Irvine, CA). PKCδ antibody was purchased from BD Biosciences (San Jose, CA) and the GFP antibody from Novus Biologicals (Littleton, CO). Gö6983 and Gö6976 were purchased from Calbiochem (San Diego, CA).

Cell culture and transfection. HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 μg/mL streptomycin, 50 U/mL gentamicin at 37°C in 5% CO2. HEK293T cells were seeded in 150 mm culture dishes and transfected 24 hours later using calcium phosphate precipitation (Clontech, Mountain View, CA).

Coimmunoprecipitation and immunoblot analysis from HEK293T cells. To identify candidate PKC-interacting proteins that might be regulated by EtOH, HEK293T cells expressing FLAG-PKCγ, HA-PKCδ or empty vector were incubated in the presence or absence of EtOH (100 mM/10 min). Cells were harvested in PBS, centrifuged at 200 x g for 10 min and the pellets homogenized in 1 ml homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, 30 mM NaF, 20 mM sodium pyrophosphate) supplemented with MiniComplete™ protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The samples were centrifuged at 20,000 x g for 30 min at 4°C and the supernatant was discarded. The pellet was resuspended and homogenized with 1 mL solubilization buffer containing 1% Triton X-100 and supplemented with MiniComplete™ protease inhibitor cocktail. The homogenate was centrifuged for 5 min at 9,000 x g and the supernatant used as the “solubilized” membrane fraction. HA-PKCδ or FLAG-PKCγ were immunoprecipitated from the solubilized fraction using either HA-agarose gel or FLAG-M2 gel (Sigma, St. Louis, MO), respectively and rotated overnight at 4°C. To identify candidate PKC interacting proteins, isozyme-specific immunoprecipitates were washed three times with PBS then sent to Applied Biomics (Hayward,
CA) for two-dimensional differential in-gel electrophoresis (2D-DIGE). Briefly, the samples were covalently linked to CyeDye fluor and separated by isoelectric focusing in the first dimension and SDS-PAGE in the second dimension. Image analysis was performed using DeCyder software and selected spots were picked for mass spectrometry analysis (MALDI/TOF/TOF).

To verify association of the PKC isozymes or the D₁R with RanBP10 and RanBP9, HEK293T cells coexpressing HA-PKCδ, FLAG-PKCγ or FLAG-D₁R with vector, RanBP10-GFP or RanBP9-GFP were subjected to coimmunoprecipitation experiments as described above. The resulting immunoprecipitates were washed three times with PBS, resuspended in SDS-PAGE sample buffer and incubated for 1 hr at 37°C. Proteins were separated electrophoretically using 4-12% Bis-Tris NuPage gels/MOPS buffer (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen). Membranes were blocked in SuperBlock (Thermo, Waltham, MA) overnight followed by incubation with primary antibodies. The blots were washed three times with TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20), incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized using SuperSignal Chemiluminescence Kit (Thermo). The optical density of the immunoblots was quantified by ImageJ (U.S. National Institutes of Health, Bethesda, MD).

**In vitro PKC assays.** HEK293T cells co-expressing FLAG-PKCγ or HA-PKCδ with either vector, RanBP10-GFP or RanBP9-GFP were coimmunoprecipitated from the solubilized membrane fraction as described above. Immunoprecipitates were centrifuged at 9,000 x g for 1 min at 4°C and the pellets were washed three times with PBS. Each pellet was resuspended with 70 μL PBS. Kinase assays were performed using a PKC Assay Kit (Upstate, Lake Placid, NY) as described previously (Rex et al., 2008). Briefly, the kinase activity in 10 μL of each sample was assayed by measuring the transfer of 32P from [γ-32P]ATP to a specific substrate peptide (Upstate, Lake Placid, NY). The remaining cell lysates were retained and used to determine PKC expression for each transfection. PKC activity was measured in an assay consisting of Assay Dilution Buffer II (ADBII end concentration- 3.33 mM MOPS [pH 7.2], 4.2 mM β-glycerol phosphate, 0.17 mM sodium orthovanadate, 0.17 mM dithiothreitol, 0.12 mM CaCl₂), 83 μM PKC substrate peptide, 0.3 μM PKA inhibitor peptide, 3.3 μM CaMK inhibitor, PKC...
lipid activators (80 ng/μL phosphatidyl serine (PS) and 8 ng/μL diacylglycerol (DAG)).
Addition of EtOH was made from a concentrated stock solution. Maximal PKC activity was
achieved in the presence of PS and DAG. Basal activity was measured in the presence of 0.5
mM EGTA instead of PS and DAG. Non-specific activity was determined in the absence of
substrate peptide. Each condition was performed in duplicate. Kinase reactions were initiated by
the addition of 10 μCi [γ-32P]ATP in Mg2+/ATP cocktail and incubated for 10 min at 30°C. The
reactions were terminated by transferring 25 μL of the mixture to P81 filter papers-followed by
washing three times with 0.75% phosphoric acid and once with acetone. The amount of
phosphorylated peptide was determined by scintillation counting and expressed as the percentage
of control. To compare PKC activity between the three groups (PKCγ + vector, PKCγ +
RanBP10 and PKCγ + RanBP9) the remaining cell lysates were subjected to Western blot and
densitometry analysis (ImageJ) to determine the relative amounts of PKC protein. To account
for any changes in PKC expression between each cotransfection, the kinase activity was divided
by the relative PKC expression (arbitrary units).

cAMP accumulation assays. Transfected HEK293T cells were seeded into 24-well plates
coated with poly-D-lysine. Duplicate wells were exposed to dopamine dilutions that were
prepared in 20 mM HEPES buffered DMEM supplemented with 200 μM sodium metabisulfite
and 30 μM RO-20-1724 (a phosphodiesterase inhibitor) (Sigma, St. Louis MO). Basal activity
was determined in the absence of dopamine. The plates were incubated at 37°C for 20 min. The
reaction was terminated by removal of the medium and addition of 3% perchloric acid to each
well for 30 min on ice. Each reaction was neutralized by addition of 15% KHCO3. cAMP
accumulation was measured using the method described by Watts and Neve (Watts and Neve,
1996)) based on the competitive binding of cAMP to PKA.

Radioligand binding assays. D1 receptor-transfected HEK293T cells were harvested in
Ca2+/Mg2+-free EBSS supplemented with 5 mM EDTA and centrifuged at 200 x g for 10 min.
Cells were lysed in a dounce homogenizer in 5 mM Tris-HCl, pH 7.4 (at 4°C) and 5 mM MgCl2.
The lysate was centrifuged at 20,000 x g for 30 min and the pellet resuspended in 50 mM Tris-
HCl (crude membrane fraction). A portion of the membrane suspension was quantified using the
BCA protein assay kit. The membrane fraction (100 μL) was added to tubes in triplicate
containing [³H]-SCH23390 (Perkin Elmer, Boston, MA) at a range of concentrations. Non-specific binding was determined in the presence of (+)-butaclamol (3 μM). Assay tubes were incubated at room temperature for 1.5 hours then terminated by rapid filtration through GF/C filters pretreated with 0.6% polyethyleneimine. Bound radioactivity was quantified by liquid scintillation counting.

**In-situ phosphorylation assays.** These assays were performed as previously described (Rankin et al., 2006). Briefly, transfected HEK293T cells were seeded into 6 well plates coated with poly-D-lysine. A portion of the transfection was retained in a 100 mm dish for radioligand binding assay to quantify the expression of D₁ receptor expression. Forty-eight hours post-transfection, the medium from each well was replaced with phosphate-free medium supplemented with 10% FBS, 50 U/mL penicillin, 10 μg/mL gentamicin and incubated for 1 hour. The medium was replaced with 1 mL phosphate-free DMEM containing 106 μCi/mL [³²P] orthophosphoric acid (Perkin Elmer, Waltham, MA) for 60 min. PKC inhibitors (Gö6983, Gö6976 at 10 μM) were included during this incubation period for select samples. Subsequently, specific wells were challenged with basal medium or media containing 10 μM dopamine for 10 min. The cells were placed on ice, rinsed with ice-cold EBSS and lysed with solubilization buffer supplemented with MiniComplete™ protease inhibitor cocktail for 1 hour at 4°C. Cell lysates were cleared by centrifugation and protein concentration quantified using the BCA protein assay kit. D₁ receptor expression was determined by radioligand binding assays using cells seeded in the 100 mm dish. Equal amounts of D₁ receptor for each condition were incubated with anti-FLAG-M2 agarose gel overnight, washed three times and resolved using 4-12% Bis-Tris NuPage gels using MOPS buffer. Dried gels were subjected to autoradiography.

**Immunofluorescence and confocal studies of proximal tubule cells in rat kidney.**
Normotensive 12 week old Wistar-Kyoto rats were anesthetized with pentobarbital (50 mg/kg IP). The kidneys were perfused with normal saline until the effluent was clear. Thin sections (3 μm) of formalin-fixed and paraffin-embedded rat kidney were deparaffinized in xylene and then rehydrated with step-down concentrations of ethanol. After antigen retrieval with citrate buffer, the D₁ receptor was visualized using a monoclonal mouse anti-D₁ receptor antibody (1:100; Novus, CO) followed by Alexa Fluor 568-donkey anti-mouse IgG antibody (red; Invitrogen).
RanBP9 was visualized using a polyclonal goat anti-RanBP9 antibody (1:100; Santa Cruz, CA) followed by Alexa Fluor 488-donkey anti-goat IgG antibody (green; Invitrogen). As a negative control, the primary antibodies were replaced with normal mouse, rabbit or goat IgG at an appropriate dilution. The slides were mounted in Vectashield Mounting Medium with 4’, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, CA). The immunofluorescent images were acquired using Zeiss 510 confocal laser scanning microscope (Zeiss, Germany). Colocalization of D1 receptor and RanBP9 is indicated by the development of a yellow color in the merged images.

**D1 receptor and RanBP9 co-immunoprecipitation from kidney cells.**

Immortalized renal proximal tubule cells (RPT, passage ~20) from adult normotensive adult humans and Wistar-Kyoto rats were used for co-immunoprecipitation (co-IP). The RPT cells were cultured at 37°C in 95% air/5% CO2 atmosphere in DMEM/F-12 with transferrin (5 µg/mL), insulin (5 µg/mL), epidermal growth factor (10 ng/mL), dexamethasone (4 µg/mL), and 5% fetal bovine serum (FBS) in a 100-mm Petri dish. The cells (80% confluence) were made quiescent by incubation for 2 hr in medium without FBS before the treatment with a D1-like receptor agonist, fenoldopam (Fen, 1 µmol/L, 10 min). The cells were lysed with ice-cold lysis buffer for 1 hr and centrifuged at 16,000 x g for 30 min. Equal amounts of cell lysate proteins (supernatant; 500 µg) were used for co-IP. Co-IP was performed according to the manufacturer’s immunoprecipitation protocol (Santa Cruz). Briefly, 2 µg of rabbit D1 receptor antibody (D1R 408), the specificity of which has been established (Yu, et al., 2006), were incubated with IP matrix at 4°C on a rotator for 1 hr, and then the complex was incubated with 500 µg lysate protein at 4°C overnight. The immunoprecipitate was pelleted and washed four times with phosphate-buffered saline. The pellet was re-suspended in sample buffer, boiled, and immunoblotted with the rabbit RanBP9 antibody. To determine the specificity of the bands, normal rabbit IgG was used as negative control and cell lysate (immunoblot) was used as positive control.

**Identification of RanBP9 expression in D1 receptor-expressing neurons in mouse striatum.**

Immunohistochemical localization of RanBP9 and RanBP10 was performed to determine possible co-expression in neurons in the striatum that express the D1 receptor. Coronal brain
sections were processed from mice in which enhanced green fluorescent protein (EGFP) is expressed under the control of the D1 dopamine receptor gene promoter (Gong et al., 2007). Frozen cut coronal sections of brains from animals that had been perfusion fixed with 4% formaldehyde, post-fixed overnight and saturated with 20% sucrose were incubated overnight with dilutions of either rabbit monoclonal antibodies directed against RanBP9 (1:100, Eptiomics, Burlingame CA, catalog number: 1928) or RanBP10 (1:100, Schulze et al., 2008). Visualization of RanBP9 and RanBP10 primary antibody localization was determined with affinity purified Cy-3 conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch, product number: 111-165-144), while EGFP may be visualized directly under epi-fluorescence illumination.

**Data analysis.** For immunoblots and phosphorylation assays, the relative intensities of bands on the autoradiographs were determined by scanning and analyzing the bands using ImageJ. Figures depict representative graphs or autoradiographs for each experimental condition. Where shown, data are presented as the mean ± SEM. All statistical analyses were performed with a level of significance established at p<0.05. Statistical analyses were conducted using GraphPad Prism 4 (GraphPad Prism Inc., San Diego, CA) software.
RESULTS

Identification of PKC\(\gamma\) and PKC\(\delta\)-interacting proteins. We previously reported that EtOH treatment decreased the kinase activities of membrane-associated PKC\(\gamma\) and PKC\(\delta\) under lipid-activated conditions (Rex et al., 2008). In contrast, cytosolic PKC\(\gamma\) and PKC\(\delta\) activities were unaffected by EtOH (Rex et al., 2008). One hypothesis for the membrane specificity of this effect is that PKC isozyme-specific interacting proteins exist in the plasma membrane and impart EtOH-sensitivity to PKC\(\gamma\) and PKC\(\delta\), or are the targets of EtOH themselves. To address this hypothesis, we utilized a PKC isozyme-specific co-immunoprecipitation approach coupled to mass spectrometry sequence analysis in order to identify novel PKC\(\gamma\) and PKC\(\delta\) interacting proteins, including those that might be regulated by EtOH.

The proteomics approach that we used is illustrated in Fig. 1. FLAG-tagged PKC\(\gamma\) was immunoprecipitated from solubilized membrane fractions prepared from HEK293T cells that were either untreated or treated with EtOH for 15 min. As a control, untransfected HEK293T cells were used for immunoprecipitation with anti-FLAG agarose beads. Similarly, HA-tagged PKC\(\delta\) was immunoprecipitated from transfected cells using HA-agarose beads under identical conditions as described above. The precipitates for each condition (control, PKC\(\gamma\), and PKC\(\delta\)) were covalently separately with fluorochromes (Cy2, Cy3 and Cy5) and subjected to two-dimensional differential in-gel electrophoresis (2D-DIGE). Proteins were separated by isoelectric focusing in the first dimension and SDS-PAGE in the second dimension. This method allows three samples to be run on a single gel and comparison of each sample based on the wavelength of the specific fluorochrome.

Fig. 2A,D show a comparison of immunoprecipitated proteins from control (untransfected) versus PKC-transfected cells. In this comparison, any unique proteins that are identified in the PKC-transfected samples are tentatively identified as specific interacting proteins for those PKCs. From the PKC\(\gamma\) screen, we identified seven protein spots that appeared to be specific for PKC\(\gamma\) (red spots) (Fig. 2A). Proteins that are common to both PKC and control immunoprecipitates were visualized as yellow spots and are presumably non-specific in nature. From the PKC\(\delta\) screen, we also identified seven protein spots that appeared to be PKC\(\delta\)-specific (red spots, Fig. 2D). We were also interested in identifying any immunoprecipitated proteins in
the PKC-transfected samples that increased or decreased in intensity upon treatment of the cells with EtOH. When comparing PKCγ versus PKCγ + EtOH treated samples, a single red protein spot was present only in the EtOH treated sample (Fig. 2B). Furthermore, this protein spot was not present in control (untransfected) cells, suggesting that the association of this protein with PKCγ might be regulated by EtOH (Fig. 2C). We identified five additional protein spots from the PKCδ + EtOH treated samples that also appeared to be PKCδ specific, one of which might be upregulated by EtOH treatment (red spot, Fig. 2E). All of the circled protein spots in Fig. 2 were excised and subjected to mass spectrometry sequence analysis.

The candidate PKC isozyme-specific interacting proteins were identified based on comparing peptide sequences derived from the mass spectrometry analyses with protein sequences submitted to the NCBI database. In some instances a single spot on the gel corresponded to multiple parent proteins. For the PKCγ screen, eight protein spots were sequenced that corresponded to 31 different parent proteins, however, in many instances only a single peptide sequence for that protein was identified. In general, we only considered proteins as bona-fide if they were identified through multiple matching peptide fragments. Table I shows a list of such proteins obtained from the PKCγ and PKCδ proteomic screens. A variety of different proteins were identified ranging from scaffolding proteins to other protein kinase C isozymes. Interestingly, heat-shock protein-70 (Hsp-70) was the only candidate PKC interacting protein that was common to both PKC screens. Heat-shock proteins assist in protein folding and assembly and are frequently observed in proteomic screens, usually as non-specific interactors (unpublished observations).

Based on the sequence data for the protein spot excised from the PKCγ + EtOH treated sample (Fig. 2B), 11 non-overlapping peptide fragments were identified that correspond to the ephrin receptor EphB1 (Holder and Klein, 1999). Additional peptide fragments identified from this protein spot corresponded to the tripartite motif protein, TRIM5 (Johnson and Sawyer, 2009). From the PKCδ screen, the single spot identified in the EtOH-treated samples (Fig. 2E) corresponds to mono-ADP-ribosyltransferase (ART3) (Okada et al., 2008). While the potential regulation of these three proteins by EtOH, and their interactions with PKCγ and PKCδ, should be investigated further, our initial assumption is that they are not responsible for the unique membrane-specific regulation of PKCγ and PKCδ activities observed previously (Rex et al., 2008), as none of them were identified in both PKC screens. Thus, while continuing to pursue
the mechanism of EtOH-mediated regulation of PKCγ and PKCδ activities, we chose to initially focus on characterizing other candidate PKC-interacting proteins that might be especially relevant to regulating D₁ receptor function. One such protein, RanBP10, and a closely related protein, RanBP9, were chosen for further characterization as RanBP9 has been shown to interact with other GPCRs (see below).

**RanBP10 and RanBP9 interact with PKCγ and PKCδ.** RanBP10 was among the candidate PKCγ-interacting proteins from our proteomic screen (Fig. 2A, Table 1). Importantly, seven non-overlapping peptide fragments of RanBP10 were identified by mass spectrometry sequence analysis, all of which span the majority of the protein sequence (Table 2). RanBP10 is a 67 kDa scaffolding protein that is expressed in a variety of tissues including the brain (Haase et al., 2008; Schulze et al., 2008; Wang et al., 2004) and is known to interact with several proteins that include the protein Ran and the receptor protein tyrosine kinase for hepatocyte growth factor, MET (Wang et al., 2004). Interestingly, there is a closely related protein, RanBP9 that has high sequence similarity to RanBP10 (Wang et al., 2004) and has been found to associate with several GPCRs including the mu-opioid receptor and the metabotropic glutamate receptors mGluR2 and mGluR8, as well as with MET (Seebahn et al., 2008; Talbot et al., 2009; Wang et al., 2004). Fig. 3 shows a structural comparison of RanBP9 and RanBP10 highlighting several conserved protein-protein interaction and functional domains such as SPRY (dual-specific kinase splA and ryanodine receptor), LisH (lissencephaly type-1-like homology), Ran protein binding domain and GEF (guanine nucleotide exchange factor) domain (Schulze et al., 2008). Recently, a β-tubulin binding domain has been localized to the N-terminal region of RanBP10 (Schulze et al., 2008). One major difference between RanBP10 and RanBP9 is the presence of a poly proline/glutamine (PQ) tract at the N-terminus of RanBP10 (Fig. 3), the role of which is unclear.

In order to initially verify the interaction of RanBP10 with PKCγ, we utilized a co-immunoprecipitation approach as shown in Fig. 4. HEK293T cells were cotransfected with FLAG-PKCγ and either empty vector or RanBP10-GFP. FLAG-PKCγ was immunoprecipitated from solubilized membrane fractions, followed by one-dimensional SDS-PAGE electrophoresis and transference to PVDF membranes. The association of RanBP10 with PKCγ was visualized by Western blot analysis. Additionally, the effect of EtOH treatment on the association of RanBP10 with PKCγ was examined. For these experiments, cells were pretreated with EtOH for
15 min prior to the preparation of the membrane fraction and subsequent immunoprecipitation. Importantly, we confirmed the interaction of RanBP10 with PKCγ using this approach (Fig. 4A). Further, the RanBP10-PKCγ association was not modulated by EtOH treatment. These experiments were also extended to examine the possible association of RanBP10 with PKCδ, even though RanBP10 was not identified in the initial PKCδ proteomic screen. Interestingly, RanBP10 was also found to coimmunoprecipitate with PKCδ and this interaction does not appear to be modulated by EtOH either (Fig. 4B).

Based on the high amino acid sequence similarity of RanBP10 and RanBP9, we wondered if RanBP9 might also associate with PKCγ or PKCδ. As shown in Supplemental Fig. 1, RanBP9 was also found to coimmunoprecipitate with both PKCγ and PKCδ, but EtOH treatment had no significant effect on this interaction.

**Effect of RanBP10 and RanBP9 on PKCγ and PKCδ kinase activities.** Although EtOH treatment did not appear to alter the association of either RanBP10 or RanBP9 with PKCδ or PKCγ (Figs. 4, Supplemental Fig. 1), given that both RanBP proteins interacted with both PKCs, we wondered if RanBP10 and/or RanBP9 could still somehow be involved in the EtOH-mediated decrease in PKC activity. To directly test this possibility, we utilized *in vitro* kinase assays to evaluate PKC activities as we had performed previously (Rex et al., 2008). Our working hypothesis was that, if either RanBP9 or RanBP10 were involved in imparting EtOH sensitivity to the PKCs, then over-expressing either RanBP protein might enhance the EtOH inhibition. PKCδ or PKCγ were coexpressed with RanBP10, RanBP9, or empty vector in HEK293T cells, followed by immunoprecipitation of the PKCs from a solubilized membrane preparation. The PKC immunoprecipitates were then used directly in the kinase assays. As we determined previously (Rex et al. 2008), the addition of EtOH to the kinase assays decreased the lipid-activated PKCγ and PKCδ activities without affecting basal enzyme activities (Fig. 5A,C). In contrast, we observed that over-expression of either RanBP9 or RanBP10 blocked the EtOH-mediated inhibition of PKC activities without affecting basal or lipid-activated activities (Fig. 5). Taken together, these results suggest that neither RanBP9 nor RanBP10 impart sensitivity of PKCγ or PKCδ to inhibition by ethanol, but rather they can inhibit this response, perhaps by blocking the association of the PKCs with another distinct interacting protein.
RanBP10 and RanBP9 associate with the D1 receptor. Although RanBP10 and RanBP9 do not appear to mediate the EtOH-induced attenuation of PKCγ and PKCδ kinase activities, they could function as important PKC scaffolding proteins. In fact, a role for RanBP10 and RanBP9 as scaffolding proteins has been well described (Haase et al., 2008; Talbot et al., 2009; Wang et al., 2004). In particular, both Ran10 and RanBP9 have been reported to associate with receptors expressed at the plasma membrane that include the MET tyrosine kinase receptor and the mu-opioid receptor, respectively (Talbot et al., 2009; Wang et al., 2004). This was of particular interest as the mu-opioid receptor is a G protein-coupled receptor as is the D1 dopamine receptor. Further, the D1 receptor is known to be phosphorylated by PKC (Gardner et al., 2001; Rex et al., 2008), which is, at least partially, mediated by PKCδ and PKCγ (unpublished observations). We hypothesized that either RanBP10 or RanBP9 might also associate with the D1 receptor and function as a scaffolding protein, perhaps to position PKCδ or PKCγ in close proximity to the receptor. To examine this possibility we used coimmunoprecipitation analyses to determine if either RanBP9 or RanBP10 could associate with the D1 receptor (Fig. 6). HEK293T cells were transfected with a FLAG-D1R construct and either RanBP10, RanBP9, or empty vector. A crude membrane preparation was prepared and the D1 receptor was immunoprecipitated from the solubilized membrane preparation, separated by one-dimensional SDS-PAGE, and transferred to PVDF membranes. The presence of the D1 receptor and both RanBP proteins were detected by Western blotting. Interestingly, both RanBP10 and RanBP9 were found to specifically coimmunoprecipitate with the D1 receptor (Fig. 6). These results suggest that RanBP9 and RanBP10 are indeed associated with the D1 receptor when expressed in HEK293T cells.

RanBP9 associates with the D1 receptor in kidney tissue. We were interested in examining the association of RanBP9 and RanBP10 with the D1 receptor in endogenous expressing tissues. Unfortunately, only the RanBP9 antisera proved useful in kidney tissue. To determine if RanBP9 and the D1 receptor associate endogenously, we first examined the cellular localization of these proteins in the rat kidney proximal convoluted tubule, where the D1 receptor is expressed (Fig. 7). Both RanBP9 and the D1 receptor were observed to be co-localized in the brush border/apical membrane of the proximal convoluted tubule of the rat kidney (Fig. 7). The D1 receptor is abundantly expressed in the proximal tubule of humans and rodent kidneys where
it negatively regulates renal sodium transport at the brush border/apical and basolateral membrane (Zeng et al 2008). We next attempted to co-immunoprecipitate RanBP9 and the D1 receptor from renal proximal tubule cells. Fig. 8 shows an experiment where the D1 receptor was immunoprecipitated from solubilized renal proximal tubule cells, resolved by one-dimensional SDS-PAGE, and the blotted with antisera directed against RanBP9. Lane 1 shows that a protein corresponding in size to RanBP9 is present in the immunoprecipitates, but absent if normal rabbit IgG is used instead of the RanBP9 antisera (Fig. 8, lane 3). This experiment was also performed subsequent to treating the cells with a D1 agonist, however, this did not alter the association of RanBP9 with the D1 receptor (Fig. 8, lane 2). Taken together, these experiments show that RanBP9 and the D1 receptor endogenously associate in the kidney.

RanBP9/10 and the D1 receptor exhibit cellular co-localization in brain tissue. We were also interested in examining the association of RanBP9/10 and the D1 receptor in the brain. In this study, immunohistochemical methods demonstrated widespread RanBP9 and RanBP10 immunoreactivity in the forebrain, including the majority of neurons in the cerebral cortex, olfactory bulb and striatum. This pattern of distribution of RanBP9 and RanBP10 immunoreactivity matches that reported for the mRNA for these genes as illustrated in the Allen brain atlas (http://www.brain-map.org/). In the striatum, half of the medium spiny neurons express the D1 receptor, which were visualized directly by their expression of EGFP in the brains of mice used for immunohistochemical localization in this study (Gong et al., 2007). As shown in Fig. 9, immunoreactivity for both RanBP9 (top panels) and RanBP10 (bottom panels), was present in striatal neurons. Also, both antigens in were present in D1-EGFP positive neurons (yellow arrows) and D1-EGFP negative neurons (blue arrows). These data demonstrate that both RanBP9 and RanBP10 co-localize in striatal neurons expressing the D1 receptor.

RanBP10 and RanBP9 decrease D1 receptor signaling. To determine if RanBP9 or RanBP10 association with the D1 receptor exhibits functional consequences, we examined D1 receptor-stimulated cAMP accumulation in the absence or presence of RanBP9/RanBP10 over-expression (Fig. 10). Notably, coexpression of either RanBP10 or RanBP9 significantly decreased maximal DA-stimulated cAMP accumulation to approximately 45% of control (Fig. 10A). However, the potency of the response was not significantly altered (EC50 ± SEM: control, 81 ± 58; RanBP10,
93 ± 48; RanBP9, 137 ± 12 nM). To establish if the inhibitory effect of RanBP10 or RanBP9 was due to changes in D1 receptor binding activity, membranes prepared from cells expressing the D1 receptor ± RanBP10 or RanBP9 were assayed using the D1-selective radioligand, [3H]-SCH23390. Interestingly, coexpression of RanBP10 did not significantly alter D1 receptor expression whereas, coexpression of RanBP9 decreased D1 receptor binding by ~50% (Fig. 10B). These results suggest that the effect of RanBP9 on D1 receptor signaling may, at least in part, be explained by decreased receptor expression. In contrast, alterations in receptor expression cannot explain the functional effect of RanBP10 expression on D1 receptor signaling.

**RanBP10 and RanBP9 increase basal D1 receptor phosphorylation by a PKC-dependent mechanism.** We previously reported that the D1 receptor appears to be constitutively phosphorylated by PKC in the basal state and that this attenuates agonist-induced cAMP accumulation (Rex et al., 2008). We further showed that EtOH selectively attenuated the kinase activities of PKCγ and PKCδ, decreased basal D1 receptor phosphorylation and potentiates cAMP accumulation (Rex et al., 2008). Given that RanBP9 and RanBP10 associate with both PKCγ and PKCδ as well as the D1 receptor, we wondered if RanBP9 or Ran BP10 might scaffold PKCδ and PKCγ in close proximity to the D1 receptor to facilitate receptor phosphorylation. In order to test this hypothesis we examined the effect of RanBP10 and RanBP9 on D1 receptor phosphorylation under basal or agonist-stimulated conditions using an *in situ* phosphorylation assay. Interestingly, coexpression of either RanBP10 or RanBP9 with the D1 receptor significantly elevated basal D1 receptor phosphorylation (Fig. 11). As expected, DA-stimulation increased D1 receptor phosphorylation when compared to basal. Previously, we showed that the agonist-induced phosphorylation of the D1 receptor is mediated through G protein-coupled receptor kinase (GRK) pathways (Gardner et al., 2001; Rankin et al., 2006). Notably, in the presence of RanBP9 or RanBP10, the percent increase in phosphorylation in response to agonist was similar to, or slightly less, than that observed in control cells (Fig. 12), suggesting that the main effect of RanBP9 or RanBP10 was to increase the basal phosphorylation state of the D1 receptor.

We were particularly interested in the mechanisms responsible for the RanBP10- and RanBP9-mediated potentiation of basal D1 receptor phosphorylation and whether or not this might be mediated by PKCs as hypothesized. To test this, we utilized the *in situ* phosphorylation
assay described above except that the cells were treated with the PKC inhibitors, Gö6983 and Gö6976, prior to incubation in basal media. Gö6983 and Gö6976 are known to inhibit several PKC isozymes including PKCδ and PKCγ (Martiny-Baron et al., 1993; Stempka et al., 1999). As described previously (Gardner et al., 2001; Rex et al., 2008), treatment with PKC inhibitors markedly decreased basal D1 receptor phosphorylation (Fig. 12). Importantly, however, treatment with the PKC inhibitors significantly attenuated the RanBP10- and RanBP9-promoted increase in basal D1 receptor phosphorylation, with Gö6983 exhibiting a somewhat greater effect than Gö6976 (Fig. 12). Taken together, these results suggest a role for PKCs, potentially PKCδ or PKCγ, in the RanBP10- and RanBP9-dependent modulation of D1 receptor signaling.
DISCUSSION

The major finding of the current investigation is the identification of RanBP9 and RanBP10 as interacting proteins for protein kinase C isozymes γ and δ as well as for the D₁ dopamine receptor. Our proteomic screen initially identified RanBP10 as an interacting protein for PKCγ, although co-immunoprecipitation analyses subsequently revealed that both PKCγ and PKCδ were also capable of interacting with the structurally related protein RanBP9. While our initial interest was in attempting to identify interacting proteins that might impart EtOH sensitivity to PKCγ/δ, neither RanBP9 nor RanBP10 appeared to affect the kinase activities of PKCγ or PKCδ. Rather, over-expression of either RanBP protein actually blocked the EtOH mediated inhibition of PKCγ/δ activities. While other explanations are possible, it is tempting to speculate that this effect could be due to RanBP9 or RanBP10 preventing the interaction of the PKCs with another interacting protein that might be involved in the EtOH modulation.

As our over-all goal was the investigation of PKC-mediated regulation of D₁ receptor signaling, we wondered if either PKC interacting protein, RanBP9 or RanBP10, could also interact with the D₁ receptor and affect its function. Our co-immunoprecipitation analyses revealed that both RanBP proteins were indeed capable of interacting with D₁ receptor complexes when expressed in HEK293 cells. Moreover, RanBP9 and the D₁ receptor are endogenously expressed in the proximal tubule of the rat kidney and RanBP9 and the D₁ receptor were found to coimmunoprecipitate using primary cultures of human renal proximal tubule cells. We also found that both RanBP9 and RanBP10, and the D₁ receptor were partially co-localized at the cellular level within brain tissue.

Functionally, the association of RanBP9 and RanBP10 with the D₁ receptor appears to attenuate signaling. This was evident as a decrease in maximal cAMP accumulation elicited by receptor stimulation. Interestingly, over-expression of RanBP9 attenuated D₁ receptor expression whereas RanBP10 did not. This might reflect fundamental differences in the interactions of RanBP9 and RanBP10 with the receptor that will merit further investigation. While a decrease in receptor expression by RanBP9 could contribute to the attenuated cAMP accumulation, this cannot explain the functional inhibition by RanBP10 suggesting that other mechanisms must be involved. Interestingly, we found that over-expression of both RanBP9 and RanBP10 lead to an
increase in the basal phosphorylation state of the D₁ receptor. Previously we found that the D₁ receptor is constitutively phosphorylated by PKC and that this attenuates receptor signaling. (Rex et al., 2008). Based on our findings that RanBP9 and RanBP10 associate with PKCδ and PKCγ, we examined the possibility that the increase in basal D₁ receptor phosphorylation was mediated by PKC. Indeed, HEK293T cells pretreated with select PKC inhibitors blocked the RanBP-dependent increase in basal D₁ receptor phosphorylation. Taken together, we propose that RanBP9 and RanBP10 regulate D₁ receptor phosphorylation and signaling through a PKC-dependent mechanism.

The identification of RanBP9 and RanBP10 as dual interacting proteins for the D₁ receptor and PKCs is consistent with their function as scaffolding proteins. Structurally both RanBP9 and RanBP10 contain multiple functional domains and there is a growing body of evidence that they both function as scaffolding molecules in immune and neural tissues (Murrin and Talbot, 2007). Of particular interest are RanBP9’s interactions with membrane receptors, especially those within the GPCR super-family such as the mu-opioid receptor (MOR) and metabotropic glutamate receptors (mGluRs). RanBP9 associates with the Gαᵢ-coupled MOR and modulates its signaling (Murrin and Talbot, 2007). Overexpression of RanBP9 reduces MOR-stimulated ERK activation and inhibits β-arrestin-mediated receptor internalization (Talbot et al., 2009). However, in contrast to our findings with the D₁ receptor, RanBP9 does not alter MOR phosphorylation or affect its regulation of cAMP production (Talbot et al., 2009). RanBP9 also associates with mGluR2 and mGluR8 in both HEK293 cells and the synaptic layers of the retina (Seebahn et al., 2008), although the role of RanBP9 in regulating glutamatergic signaling is currently unclear (Seebahn et al., 2008). Compared to RanBP9, there is little published information regarding RanBP10-dependent modulation of signaling; however, both RanBP10 and RanBP9 are described as scaffolding proteins for MET, a membrane-bound receptor tyrosine kinase for hepatocyte growth factor (Wang et al., 2004). Association of RanBP9 with MET increases recruitment of the protein SOS to MET and enhances ERK signaling. RanBP10 also associates with MET but functions as a dominant-negative to reduce SOS recruitment and decrease ERK-mediated signaling (Wang et al., 2004).

At the cellular level, we show that RanBP9 and RanBP10 can associate with the membrane bound D₁ receptor and PKCδ/γ. These findings are consistent with the site of action of the D₁ receptor and regulation by PKCδ and PKCγ. Importantly, Denti et al., 2004 show that
endogenous levels of RanBP9 expressed in the epithelial cells of lung, kidney and breast are localized to the plasma membrane and cytoplasm. In the retina, RanBP9 is localized to the synaptic processes of cholinergic amacrine cells (Seebahn et al., 2008). In contrast, RanBP10 is expressed in multiple cellular locations including the cytoplasm and nucleus of megakaryocyte cells (Schulze et al., 2008), however, there is currently no information regarding the subcellular localization of RanBP10 in other cell types, including neurons. Collectively the subcellular locations of RanBP9 and RanBP10 appear to depend largely on the cell type.

Consistent with the notion of RanBP9 and RanBP10 functioning as scaffolding proteins, neither RanBP protein appears to modulate the kinase activities of PKC\(_\delta\) or PKC\(_\gamma\), but rather may play a role in the spatial and temporal organization of the D\(_1\) receptor-PKC signaling complex. Several PKC-interacting proteins have been described that anchor specific PKC isoforms to the appropriate signaling complex at the correct intracellular location. For example, RACK1 (receptor for activated C kinase 1) preferentially binds to PKC\(_{\beta_{ii}}\) and PKC\(_{\epsilon}\) (Besson et al., 2002; Ron et al., 1994; Stebbins and Mochly-Rosen, 2001). RACK1 is believed to stabilize the active conformation of PKC\(_{\beta_{ii}}\) and shuttle the enzyme to the correct subcellular site (Ron et al., 1999). Similarly, RACK2 associates with PKC\(_{\epsilon}\) and translocates the kinase to the Golgi membranes (Csukai et al., 1997). PICK1 (protein interacting with C kinase 1) is a scaffolding protein that associates with presynaptic mGluR7 and PKC. Notably, PICK1 is required for the PKC-dependent phosphorylation of mGluR7 and for stable receptor expression at the cell surface (Suh et al., 2008). Importantly, mGluR7-dependent neuronal plasticity is impaired in mice lacking PICK1 (Suh et al., 2008).

In summary, we report RanBP10 and RanBP9 as novel scaffolding proteins for the D\(_1\) receptor, PKC\(_\delta\) and PKC\(_\gamma\). We speculate that these dual specificity scaffolding proteins may function as signaling integrators and dictate the efficient regulation of D\(_1\) receptor signaling by PKC\(_\delta\) and PKC\(_\gamma\).
ACKNOWLEDGMENTS

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REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Figure 1. Experimental scheme for identifying candidate PKC-interacting proteins. FLAG-PKCγ or HA-PKCδ constructs were expressed in HEK293T cells whereas untransfected cells served as controls. The cells were either untreated or pretreated with ethanol (100 mM/15 min), disrupted, and the PKC constructs were immunoprecipitated (IP) from the solubilized membrane fractions using antisera to either the FLAG or HA tags, as described in the Materials and Methods. The immunoprecipitates were then labeled with CyDYE™ fluors as follows: untransfected cells, Cy2; PKC-transfected, Cy3; PKC transfected plus ethanol treatment, Cy5. The labeled proteins were then resolved by 2D-gel electrophoresis and identified as described in the Materials and Methods.

Figure 2. Identification of candidate PKC-interacting proteins. Each CyDYE-labeled PKC sample was simultaneously separated on a 2D-gel and scanned at specific wavelengths to reveal each of CyDYE signals. Overlay of images reveal protein spots that are presumably specific (or non-specific) to a given sample. Circled: protein spots selected for identification by mass spectrometry. Top panel (A-C): PKCγ; bottom panel (D-F): PKCδ.

Figure 3. Structural organization of RanBP10 and RanBP9. The RanBP10 and RanBP9 proteins are aligned and compared. Conserved regions include a SPRY (dual-specific kinase splA and ryanodine receptor) domain; LisH (lissencephaly type-1-like homology) domain; Ran binding protein domain and GEF (guanine exchange factor) domain. RanBP10 contains a poly proline/glutamine tract at the amino terminus that is absent in RanBP9 (adapted from Yudin and Fainzilber, 2009).

Figure 4. Association of RanBP10 with PKCγ and PKCδ. Coimmunoprecipitation and immunoblot analyses of RanBP10 with (A) PKCγ or (B) PKCδ. HEK293T cells were transfected with empty vector, FLAG-PKCγ + RanBP10-GFP or HA-PKCδ + RanBP10-GFP. PKCγ or PKCδ were immunoprecipitated from solubilized membrane fractions prepared from HEK293T cells that were either untreated or pretreated with ethanol (100 mM/15 min). As a control, the solubilized membrane fraction prepared from cells transfected with empty vector.
was incubated with either FLAG or HA-beads. After SDS-PAGE, the gels were blotted with antisera directed to GFP (top panels) or to PKCγ or PKCδ. The experiment shown is representative of three independent experiments.

**Figure 5. Association of RanBP9 with PKCγ and PKCδ.** Coimmunoprecipitation and immunoblot analysis of RanBP9 with (A) PKCγ and (B) PKCδ. HEK293T cells were transfected with either empty vector, or FLAG-PKCγ + RanBP9-GFP, or HA-PKCδ + RanBP9-GFP. PKCγ or PKCδ were immunoprecipitated from solubilized membrane fractions prepared from HEK293T cells that were either untreated or pretreated with ethanol (100 mM/15 min) as described in the Materials and Methods. As a control, the solubilized membrane fractions prepared from cells transfected with empty vector was incubated with either FLAG or HA-beads. This representative experiment was performed three times with similar results.

**Figure 6. RanBP10 and RanBP9 coimmunoprecipitate with the D1 receptor.** The FLAG-D1 receptor was expressed in HEK293T cells either alone or with RanBP10-GFP or RanBP9-GFP. Membranes were prepared and solubilized followed by immunoprecipitation of the D1 receptor using anti-FLAG antisera as described in the Materials and Methods. After resolution of the precipitates on SDS-PAGE, the gels were blotted with antisera directed to GFP (top panels) to detect RanB10 or RanBP9, or antisera directed to the D1 receptor (bottom panels). The experiment shown is representative of 3 independent experiments.

**Figure 7. D1 receptor and RanBP9 colocalization in rat kidney.** D1R (red) and RanBP9 (green) are expressed in the brush border (BB), apical membrane, and to a lesser extent observed in the cytoplasm of the proximal tubule (PT) (400 X). The immunofluorescence images were acquired using Zeiss 510 confocal laser scanning microscope (Zeiss, Germany). Co-localization of the D1 receptor and RanBP9 is indicated by the yellow color in the merged image. The experiment shown is representative of 3 independent experiments.

**Fig. 8. Co-immunoprecipitation of the D1 receptor and RanBP9 endogenously expressed in kidney tissue.** Immortalized renal proximal tubule cells (passage ~20, 80% confluent), made quiescent by incubating the cells for 2 hr in medium without FBS, were treated with vehicle
(Veh, sterile water, 10 min, lane 1) or a D1-like agonist, fenoldopam (Fen, 1 µmol/L, 10 min, lane 2). Lysates of treated cells or IgG were immunoprecipitated (IP) and immunoblotted (IB) as shown. To determine the specificity of the bands, normal rabbit IgG was used for immunoprecipitation and served as the negative control (lane 3) and Veh-cell lysate (immunoblot, lane 4) was used as positive control. The studies were performed three times with similar results.

Fig. 9. Cellular co-localization of the D1 receptor and RanBP9 in brain. Brain sections through the striatum from transgenic mice in which D1 receptors are marked with enhanced green fluorescent protein (EGFP) were processed for fluorescence immunohistochemical localization of RanBP9 (top panels) and RanBP10 (bottom panels). Immunoreactivity for both RanBP9 (RanBP-IR) and RanBP10 (RanBP-IR) co-localize with both D1-EGFP positive striatal neurons (yellow arrows) and D1-EGFP negative striatal neurons (blue arrows). Thus both RanBP9 and RanBP10 appear to co-localize with striatal neurons expressing the D1 receptor.

Figure 10. Effect of RanBP10 and RanBP9 on D1 receptor expression and signaling. (A) RanBP10 or RanBP9 were co-expressed with the D1 receptor in HEK293T cells followed by assessment of DA-stimulated cAMP accumulation. The curves on the left are representative experiments whereas the histograms on the right represent averaged data from three experiments using 100 µM DA as the stimulus. Expression of either RanBP10 or RanBP9 decreased maximal DA-stimulated cAMP accumulation when compared to control (D1 receptor + empty vector): Emax ± SEM for D1R + RanBP10 and D1R + RanBP9 were 47 ± 6% and 46 ± 10% of control, respectively. (B) Saturation radioligand binding experiments were performed on membranes prepared from control cells (D1 receptor + empty vector) or cells expressing the D1 receptor along with either RanBP10 or RanBP9. The curves on the left are representative experiments whereas the histograms on the right represent averaged Bmax data from three experiments. RanBP9 coexpression reduced the D1 receptor Bmax value to 60 ± 14% of control. (*p<0.05, **p<0.005, paired Student’s t-test). (C)

Figure 11. RanBP10 and RanBP9 increase basal D1 receptor phosphorylation. In situ phosphorylation experiments were performed on HEK293T cells expressing the D1 receptor, D1
receptor + RanBP10, and D1 receptor + RanBP9 as described in the Materials and Methods. Cells were incubated with media (control) or DA (10 μM) for 10 min. Top panel, autoradiogram of D1 receptor immunoprecipitates from a representative in situ phosphorylation assay. The lanes in the gel correspond to the bars in the bottom panel. Bottom panel, average values of band densities for each condition. The data are normalized as the percentage of control for each individual experiment. The histograms represent the mean ± SEM from four independent experiments (*p<0.05, **p<0.01, ***p<0.001, ANOVA followed by Bonferroni pair wise comparisons).

Figure 12. The RanBP9 and RanBP10-induced increase of D1 receptor phosphorylation is blocked by PKC inhibitors. HEK293T cells expressing the D1 receptor, D1 receptor + RanBP10, and D1 receptor + RanBP9 were treated with the PKC inhibitors, Gö6983 (10 μM) or Gö6976 (10 μM) for 60 min followed by the assessment of basal phosphorylation using an in situ phosphorylation assay as described in Fig. 11. Top panel, autoradiogram of D1 receptor immunoprecipitates from a representative in situ phosphorylation assay. The lanes in the gel correspond to the bars in the bottom panel. Bottom panel, average values of band densities for each condition. The data are normalized as the percentage of control for each individual experiment. The histograms represent the mean ± SEM from three independent experiments. (ANOVA followed by Bonferroni pair-wise comparisons, *p<0.05, **p=0.056).
TABLE 1

Candidate PKCγ and PKCδ interacting proteins identified using mass spectrometry analysis

Proteins identified based on MS/MS analysis of selected protein spots highlighted in Fig. 2.

<table>
<thead>
<tr>
<th>Candidate PKCγ- interacting proteins</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAC/CDC42 exchange factor (GEFT)</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>Ran-binding protein 10 (RanBP10)</td>
<td>Scaffolding protein</td>
</tr>
<tr>
<td>Hsp-70*</td>
<td>Protein folding/stability</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Cell structure/communication</td>
</tr>
<tr>
<td>PKCα</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>Ephrin receptor EphB1#</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>Tripartite motif protein (TRIM5)#</td>
<td>Protein localization</td>
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<tr>
<td>Voltage-gated sodium channel subtype III</td>
<td>Cell signaling</td>
</tr>
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</table>

<table>
<thead>
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<th>Candidate PKCδ- interacting proteins</th>
<th>Function</th>
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</thead>
<tbody>
<tr>
<td>Cytoplasmic dynein</td>
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<tr>
<td>Hsp-70*</td>
<td>Protein folding/stability</td>
</tr>
<tr>
<td>Hsp-90</td>
<td>Protein folding/stability</td>
</tr>
<tr>
<td>IP3 receptor type 1</td>
<td>Ca2+ signaling</td>
</tr>
<tr>
<td>Microtubule-actin crosslinking factor</td>
<td>Cell structure</td>
</tr>
<tr>
<td>Mono-ADP-ribosyltransferase (ART3)#</td>
<td>Protein modification</td>
</tr>
<tr>
<td>Valosin-containing protein</td>
<td>Regulation of cellular activities</td>
</tr>
</tbody>
</table>

*Common to both PKCγ and PKCδ proteomics screens.

#Identified in EtOH-treated samples only.
TABLE 2

RanBP10 peptides identified in co-immunoprecipitation-mass spectrometry experiments

The protein spot indicated in Fig. 2A was picked and subjected to mass-spectrometry sequencing. The seven peptides shown correspond to fragments of RanBP10.

<table>
<thead>
<tr>
<th>Trypsin-digested peptides</th>
<th>Location (RanBP10)*</th>
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<tbody>
<tr>
<td>LYPAVNQQETPLPRSWSPK</td>
<td>42-60</td>
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<tr>
<td>YNYIGLSQGNLRVHYK</td>
<td>63-78</td>
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<tr>
<td>ATHPIPAACGIYYFEVK</td>
<td>92-108</td>
</tr>
<tr>
<td>GRDGYMGIGLSAQQVMNRLPGWDK</td>
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<tr>
<td>QFVEMVNGTDSEVR</td>
<td>336-349</td>
</tr>
<tr>
<td>RQLCGGNQAAETER</td>
<td>499-510</td>
</tr>
<tr>
<td>EPVCAALNSAILESQNLPK</td>
<td>566-584</td>
</tr>
</tbody>
</table>

*Indicates location based on multiple alignments of the peptide fragments with RanBP10. Full-length RanBP10 is 620 amino acids.
Figure 1

1. Untransfected IP labeled with Cy2
2. PKC IP labeled with Cy3
3. PKC + EtOH IP labeled with Cy5

4. Mix labeled extracts
5. Separated by 2D-DIGE
6. In-gel analysis of differences
7. Spot picking and MALDI-ToF mass spectrometry
Figure 3

β-tubulin-binding domain

RanBP10

Ran-binding domain

GEF domain

PQ-rich region

RanBP9
**Figure 5**

**A. PKCγ - lipid activated**

**B. PKCγ - basal**

**C. PKCδ - lipid activated**

**D. PKCδ - basal**
Figure 11

Band Density (% control)

- **D_{1}R**
- **D_{1}R+RanBP10**
- **D_{1}R+RanBP9**
- **D_{1}R**
- **D_{1}R+RanBP10**
- **D_{1}R+RanBP9**

Basal vs. DA stimulation comparison.
Figure 12

[Image of a bar chart showing band density (% control) for different conditions with statistical comparisons indicated by asterisks (*) and double asterisks (**) for significant differences.](image-url)