Association of the D2 Dopamine Receptor Third Cytoplasmic Loop with Spinophilin, a Protein Phosphatase-1-interacting Protein*

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Signaling through D2 class dopamine receptors is crucial to correct brain development and function, and dysfunction of this system is implicated in major neurological disorders such as Parkinson's disease and schizophrenia. To investigate potential novel mechanisms of D2 receptor regulation, the third cytoplasmic loop of the D2 dopamine receptor was used to screen a rat hippocampal yeast two-hybrid library. Spinophilin, a recently characterized F-actin and protein phosphatase-1-binding protein with a single PDZ domain was identified as a protein that specifically associates with this region of D2 receptors. A direct interaction between spinophilin and the D2 receptor was confirmed *in vitro* using recombinant fusion proteins. The portion of spinophilin responsible for interacting with the D2 third cytoplasmic loop was narrowed to a region that does not include the actin-binding domain, the PDZ domain, or the coiled-coil. This region is distinct from the site of interaction with protein phosphatase-1, and both D2 receptors and protein phosphatase-1 may bind spinophilin at the same time. The interaction is not mediated via the unique 29-amino acid insert in D2$_{long}$; both D2$_{long}$ and D2$_{short}$ third cytoplasmic loops interact with spinophilin *in vitro* and in yeast two-hybrid assays. Expression of D2 receptors containing an extracellular hemagglutinin epitope in Madin-Darby canine kidney cells results in co-localization of receptor and endogenous spinophilin as determined by immunocytochemistry using antibodies directed against spinophilin and the HA tag. We hypothesize that spinophilin is important for establishing a signaling complex for dopaminergic neurotransmission through D2 receptors by linking receptors to downstream signaling molecules and the actin cytoskeleton.

Dopamine receptors are members of the G protein-coupled receptor (GPCR)[1] superfamily that mediate their actions by coupling to intracellular signal transduction cascades via heterotrimeric G proteins. Five different dopamine receptors have been described belonging to two classes that are defined based on structure, function, and pharmacology (1). D2-like receptors, D2, D3, and D4, couple to G proteins of the $G_{i/o}$ class and have diverse downstream effectors, including adenylyl cyclase and G protein-coupled inward rectifier K^+ channels (GIRKs). In contrast, D1-like receptors D1 and D5 are coupled to increases in adenylyl cyclase activity through G_s. D1-like receptors have short third cytoplasmic loops (*i3*) and very long carboxyl-terminal cytoplasmic tails. Conversely, D2-like receptors are characterized by long third cytoplasmic loops and short carboxyl-terminal tails. D2-like receptors are more polymorphic than D1-like receptors; for example, D2 exists as two isoforms, D2$_{long}$ and D2$_{short}$, which differ by 29 amino acids within the third cytoplasmic loop (2). In many GPCRs the third cytoplasmic loop is important for the coupling to G proteins. Typically, short peptide sequences near the amino and carboxyl termini of the third loop participate in the coordination of receptor-G protein interactions (3–5). Thus, the rest of the third cytoplasmic loop might then be free to interact with cytosolic or membrane-associated proteins that regulate receptor-mediated signaling cascades (6).

The complexity of signaling pathways and the specificity of effector action generated by G protein-coupled receptors suggest a higher order organization than is currently known to exist (7, 8). Regulation of GPCR desensitization and resensitization through phosphorylation-dependent events mediated by G protein-coupled receptor kinases and the arrestins have been worked out for many classes of GPCRs (9). These proteins are responsible for terminating receptor-mediated signaling following ligand binding and receptor activation. Although much progress has been made to define these pathways, little is known about the basic organization of G protein-coupled receptors, their effector molecules, and regulators of receptor function into complexes that affect GPCR-mediated signaling. In this study we show that the third cytoplasmic loop of D2 dopamine receptors binds to spinophilin, a protein phosphatase-1 (PP-1)-binding protein that contains an amino-terminal actin-binding domain and a single PDZ domain (10) through a novel, non-PDZ-dependent mechanism. These data and others (10, 11) suggest that spinophilin may be a core member of a signaling complex that links D2 receptors and various downstream molecules to the actin cytoskeleton within neurons that receive dopaminergic input.

EXPERIMENTAL PROCEDURES

Construction of Bait Plasmids—cDNA fragments encoding the third cytoplasmic loop (*i3*) of D2$_{long}$ (D2L) and D2$_{short}$ (D2S) (Tyr^{213}–Lys^{370} and Tyr^{213}–Lys341, respectively) were amplified by PCR using appropriate primers containing *Sal*I and *Not*I restriction sites and rat D2L and D2S cDNA templates kindly provided by Dr. Marc Caron (Duke University). These fragments were ligated in frame to the corresponding sites in pPC97 (12), creating plasmids encoding either GAL4BD-D2L*i3* or GAL4BD-D2S*i3* fusion proteins.

Yeast Two-hybrid Assays—Two hybrid techniques were performed as described (Ref. 13; CLONTECH Manual). For screening cDNA libraries,

[1] The abbreviations used are: GPCR, G protein-coupled receptor; PP-1, protein phosphatase-1; D2L, D2$_{long}$; D2S, D2$_{short}$; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline.

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the bait vector pPC97.D2L*i3* was transformed into yeast strain CG-1945 (CLONTECH) using a modified lithium acetate protocol (14). After confirming expression of the bait protein, a rat hippocampal cDNA library in the vector pPC86 (15) was transformed into the strain harboring the bait plasmid, and transformants expressing both the bait and interacting prey proteins were selected on medium lacking leucine, tryptophan, and histidine. His$^+$ colonies were then tested for β-galactosidase activity using the filter lift assay. Clones that were consistently phenotypically His$^+$ and *lacZ*$^+$ were further characterized. Approximately 1×10^6 transformants were screened; several different clones met these criteria. A single clone was chosen for further pursuit based on its strongly His$^+$/*lacZ*$^+$ phenotype. Library plasmids were isolated using the Yeast DNA Isolation system (Stratagene) and amplified in *Escherichia coli*. Sequencing was performed by the Automated Sequencing Facility (University of North Carolina at Chapel Hill).

Construction of Bacterial Fusion Proteins and in Vitro Binding Assays—The cDNA fragment encoding spinophilin (amino acids 100–767) was excised from pPC86.clone7 as a *Sal*I-*Not*I fragment and subcloned into pGEX5X2 (Amersham Pharmacia Biotech). This plasmid, pGEX5X2.spinophilin (100–767), was transformed into *E. coli* strain BL21DE3(pLysS) (Novagen), allowing expression of spinophilin as a glutathione *S*-transferase (GST) fusion protein. Deletion constructs were made using a combination of restriction endonuclease digestion and PCR cloning. Several GST fusion proteins showed a consistent pattern of lower molecular weight bands in the purified sample. We believe these are either unstable degradation products or incompletely translated products. A GST fusion protein encoding amino acids 101–372 of neurabin (16) was generated by PCR amplification of the corresponding cDNA from rat brain Quick Clone cDNA (CLONTECH) using the Advantage GC-rich PCR kit (CLONTECH). The cDNA fragment was subcloned into pGEX5X2 as described above. Bacterial fusion protein production was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside and overnight growth at 30 °C. Fusion protein was either purified in large batches using glutathione-agarose (Sigma), or induced bacteria were frozen in small aliquots and fusion protein was purified at the time of the experiment. To generate a tagged D2L third cytoplasmic loop fusion, the *Sal*I-*Not*I fragment encoding *D2Li3* was excised from the bait plasmid and inserted into pET32c (Novagen), creating pET32c.D2L*i3*. This plasmid, encoding a fusion protein consisting of thioredoxin, an S tag, a His$_6$ tag and the third cytoplasmic loop of D2L, was transformed into BL21DE3(pLysS) cells, and protein production was induced as above. The fusion protein was purified from the insoluble fraction using Ni^{2+}-nitrilotriacetic acid-agarose (Novagen) after solubilization of inclusion bodies in 6 M urea. Fusion protein was refolded by step-wise removal of denaturant by dialysis. For binding assays, GST-spinophilin (100–767) or negative control proteins were immobilized on GSH-agarose at 4 °C in binding buffer (50 mM sodium phosphate, pH 7.4, 10% glycerol, 0.05% Triton X-100, 0.1% nonfat dry milk). His-tagged D2L*i3* were incubated with the immobilized GST fusion proteins or GST alone in 200 μl of binding buffer for 2 h at 4 °C. Proteins bound to the immobilized resin were collected by centrifugation, washed extensively in binding buffer + 150 mM NaCl, eluted in 1× Laemmli sample buffer, and separated by SDS-polyacrylamide gel electrophoresis. Unbound proteins were precipitated with acetone and analyzed separately. Proteins were transferred to Immobilon-P (Millipore) membranes, and bound (or unbound) tagged D2L*i3* was detected by incubation with S protein-horseradish peroxidase (Novagen; 1:10,000) for 1 h at room temperature. Membranes were washed 3 times in TTBS (100 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and developed using ECL (NEN Life Science Products).

In Vitro Binding with ^{35}S-Labeled Proteins—Binding assays were done essentially as described above, except that ^{35}S-labeled proteins were produced using pET28c.D2L*i3* as template in coupled *in vitro* transcription/translation reactions (TNT system, Promega). This plasmid was created by subcloning the *Sal*I-*Not*I *D2Li3* fragment into pET28c (Novagen), which lacks the thioredoxin and S protein tags and instead has a short sequence encoding a T7 and a His$_6$ tag. ^{35}S-Labeled PP-1γ1 was synthesized using pGEM-7z-rPP1γ1 (Provided by Dr. Ernest Y. C. Lee, New York Medical College) as template. 5 μl of TNT reaction product were added directly to the GST fusion protein-coupled beads in 500 μl of binding buffer. Beads were tumbled for 2 h at 4 °C and pelleted at 8000 rpm for 20 s, and supernatant was removed. Beads were washed three times with 1 ml of binding buffer plus 50 mM NaCl or 500 mM NaCl. Protein was eluted from beads in 1× Laemmli sample buffer. Proteins in the unbound fractions were precipitated and analyzed separately (data not shown). Total bound fractions were separated on 13.5% polyacrylamide gels. Dried gels were subjected to PhosphorImager analysis by exposure overnight followed by scanning on a

Storm 840 PhosphorImager (Molecular Dynamics). All binding assays were performed multiple times to confirm reliability of the *in vitro* interactions.

Antibody Production and Purification—Rabbit polyclonal antibodies were generated against two different peptides based on the primary sequence of rat spinophilin. Peptide 1 contains amino acids 114–131 (GTSVSERVSRFNSKPAPS); peptide 2 contains amino acids 367–390 (DNGRAPDMAPEEVDESKKEDFSEA). Peptides were synthesized and coupled to keyhole limpet hemocyanin at the University of North Carolina Peptide Synthesis Core Facility. Antibodies were produced commercially (Covance). Antisera were tested for their ability to recognize the expected 140-kDa protein from lysates of MDCK cells, as well as the appropriate GST fusion protein, by Western blot. We chose to use the antibody NC152, directed against peptide 1, because it gave unambiguous results by Western blot analysis and had less background than the antibody against peptide 2 (NC153). Crude serum was purified using DEAE-Affy-Gel Blue (Bio-Rad) according to the manufacturer's instructions, except that serum was diluted 1:5 in application buffer before applying to column.

Construction of HA-tagged D2S Dopamine Receptor—The tagging of D2S was based on the scheme developed by Keefer *et al.* (17) and Wozniak and Limbird (18). Briefly, PCR was used to generate a 514-base pair fragment containing a 5′ *Hin*dIII site, a Kozak site, and nucleotides encoding the HA tag/linker region (YPYDVPDYALV) inserted between Asp^2 and Pro^3 in the D2S protein sequence. The corresponding portion of the wild type receptor cDNA in pRc/RSV-hD2S (provided by Tony Sandrasagra, Hoechst-Marion Roussel) was excised and replaced with the HA tag encoding fragment. Recombinant receptor cDNAs were detected by PCR using a 5′ primer specific for the HA tag. Sequencing confirmed the presence of the HA tag in the correct reading frame.

Generation of MDCK Cells Expressing HA-D2S—MDCK type II cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 + 5% fetal calf serum. Cells were transfected with pRc/RSV.HA-hD2S using Lipofectin (Life Technologies, Inc.) according to the manufacturer's instructions. Stable transfectants were selected by culturing in media containing 400 μg/ml G418. Colonies were subcloned and checked for expression of HA-D2S by immunofluorescence microscopy using monoclonal antibody HA.11 (BabCo). Nonclonal cell lines expressing high levels of HA-D2S receptor in ~75% of cells were used for immunofluorescence microscopy.

Immunofluorescence Microscopy—MDCK cells expressing HA-D2S were plated on Transwell filters and grown to confluence (5–7 days). Cells were fixed in −20 °C methanol for 3 min. After washing with PBS, cells were blocked in PBS + 0.2% bovine serum albumin, 4% nonfat dry milk, 0.1% Triton X-100 for either 4 h at room temperature or overnight at 4 °C. The cells were then incubated with primary antibodies diluted to the appropriate concentrations in PBS + 0.2% bovine serum albumin overnight at 4 °C. Cells were washed three times for 10 min in PBS + 0.1% Triton X-100 followed by one wash with PBS. Secondary antibodies were diluted in PBS + 0.2% bovine serum albumin and applied for >2 h at room temperature. Cells were washed as above and mounted on glass slides using a Molwiol 4–88 mounting medium. Images were captured using a Leica TCS-NT confocal microscope and processed in Adobe Photoshop 4.0.

RESULTS AND DISCUSSION

In an attempt to isolate D2 dopamine receptor-associated proteins, we used the yeast two-hybrid system to identify proteins that interact with the third cytoplasmic loop. Screening of a rat hippocampal cDNA library yielded several potential interactors (His$^+$), one (clone 7) of which was chosen for further pursuit, based on its moderately strong blue phenotype in the β-galactosidase assay. The specificity of the interaction in yeast was tested by retransforming pPC86.clone7 along with the original bait pPC97.D2L*i3*, pPC97 alone, or a plasmid encoding an irrelevant bait back into yeast strain CG-1945. The interaction between the receptor loop and the novel protein specifically allowed growth on medium lacking leucine, tryptophan, and histidine (SC-Leu/Trp/His) (Fig. 1). Neither the bait protein, GAL4BD-D2L*i3*, nor the prey protein were able to activate transcription of the reporter genes in the presence of only empty prey or bait vectors, respectively (Fig. 1). Digestion of the library plasmid, pPC86.clone7, with *Sal*I and *Not*I yielded an ~2-kilobase pair cDNA fragment (data not shown). Se-
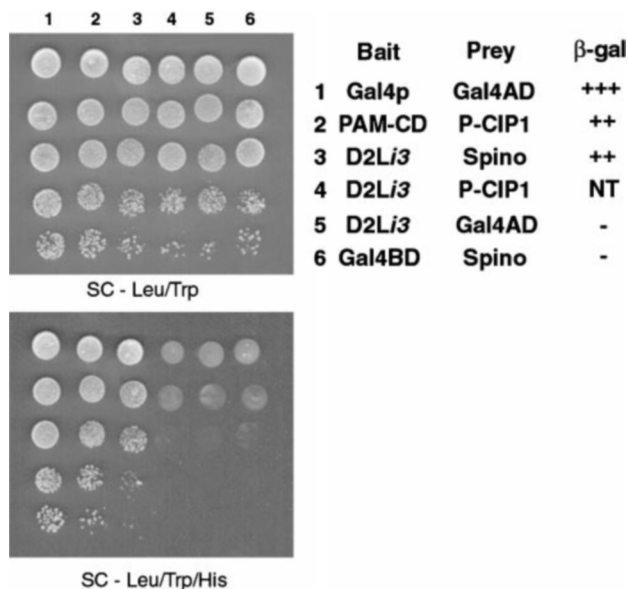
FIG. 1. **The third cytoplasmic loop of D2L (D2L*i3*) interacts with spinophilin in the yeast two-hybrid system.** Yeast strain CG-1945 was co-transformed with plasmids encoding fusions to either the GAL4 DNA-binding domain (*Bait*) or the GAL4 activation domain (*Prey*). The ability of two proteins to interact was tested by growing the yeast on selective medium (SC-Leu/Trp/His). The interaction between D2L*i3* and spinophilin is specific; the receptor loop does not interact with control proteins. Spinophilin, too, is unable to activate transcription of the reporter gene in the absence of D2L*i3*. To the *right* is indicated the relative strength of the interaction as determined by visual inspection of β-galactosidase (β-*gal*) filter lift assays. +++, very strong; ++, moderate; +, weak; −, negative; *NT*, not tested.

quencing of this fragment and analysis of the cDNA sequence using the BLAST programs at the NCBI indicated that the cDNA insert in pPC86.clone7 encoded amino acids 100–767 of the recently identified protein spinophilin. Spinophilin was originally identified in another yeast two-hybrid screen as a PP-1-interacting protein (10). Subsequently, spinophilin was also described as an actin-binding protein, based on ability to bind ^{125}I-labeled actin by blot overlay analysis, and termed neurabin-II (11). Spinophilin is an 817-amino acid protein that is expressed in many tissues but enriched in brain and contains several important structural domains. At the amino terminus, from amino acids 1–154, is a putative actin-binding domain (11). The carboxyl-terminal half of the protein contains both a single PDZ domain and a region predicted to form a coiled-coil structure (10, 11) (see Fig. 3*A*). Typically, PDZ domains bind to specific carboxyl-terminal sequences of the form E(S/T)*X*(V/I)$_{COOH}$, although there are exceptions to this consensus (19). In recent years this domain has been implicated as a common protein-protein interaction domain found in proteins that function as scaffolds for organizing ion channels, receptors, and cytoplasmic signaling components into macromolecular signaling complexes (see Refs. 19 and 20 for review). This protein was named spinophilin on the basis of its intense immunoreactivity in the head and neck of dendritic spines on hippocampal and striatal neurons. Although spinophilin is most highly concentrated in hippocampus, it also expressed at high levels in many brain regions, including caudate, putamen, and hypothalamus, with lower levels being expressed in cerebellum and cortex (10). These brain regions correspond to regions where moderate to high levels of D2 dopamine receptor expression have been observed (21–23).

To confirm the interaction between the third cytoplasmic loop of D2L and spinophilin biochemically, we used an *in vitro* binding assay to test for the direct interaction of recombinant fusion proteins. GST, GST-PAM (unrelated protein), and GST-
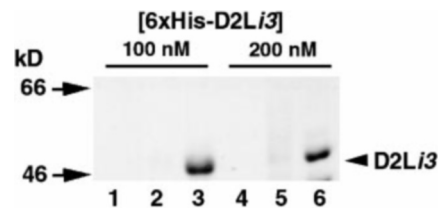

FIG. 2. **Spinophilin binds to the third cytoplasmic loop of D2L *in vitro*.** The region of spinophilin (amino acids 100–767) isolated in the two-hybrid screen was expressed as a bacterial GST fusion protein and immobilized on glutathione-agarose beads. The third cytoplasmic loop of D2L was also expressed and purified from bacteria as a hexa-histidine-tagged protein. Different concentrations of the His$_6$- D2L*i3* fusion protein were incubated with either immobilized GST alone (*lanes 1* and *4*), GST-PAM-CD (as a negative control) (*lanes 2* and *5*), or GST-spinophilin (100–767) (*lanes 3* and *6*). Bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis and blot overlay analysis with horseradish peroxidase-conjugated S protein, which recognizes a short peptide sequence in the tagged D2L*i3* fusion protein.

spinophilin (100–767) were immobilized on glutathione-agarose beads and incubated with His$_6$-tagged D2L*i3*. After extensive washing the bound fraction was analyzed by blotting with horseradish peroxidase-conjugated S protein. We found that the His$_6$-D2L*i3* fusion protein specifically bound the GST-spinophilin (100–767) fusion but not GST or GST-PAM-CD (Fig. 2), confirming our yeast two-hybrid results.

As discussed above, spinophilin has several domains that suggest specific functions. Spinophilin has been reported to bind F-actin through an amino-terminal actin-binding domain (11), as well as PP-1 through an interaction carboxyl-terminal to amino acid 296 (10). Therefore, we were interested in narrowing the site of interaction between D2L*i3* and spinophilin to a specific region of spinophilin. To do this, a series of GST fusion proteins with various regions of spinophilin were constructed and purified from bacteria (Fig. 3*A*). ^{35}S-Labeled proteins were incubated with GST fusion proteins, and bound protein complexes were isolated, washed, separated by electrophoresis, and analyzed by PhosphorImager analysis. Binding of labeled D2L third cytoplasmic loop to spinophilin only occurred with GST-spinophilin fusion proteins containing the region encompassed by amino acids 100–371 (Fig. 3*B*, *lanes 2*, *3*, and *6*). The site of interaction between spinophilin and D2L dopamine receptors occurs within the unique region distal to the actin-binding domain but upstream of the both the PDZ domain and the coiled-coil. Interestingly, the interaction occurs independently of the PDZ domain, suggesting that spinophilin links D2 receptors to other membrane-anchored proteins that contain carboxyl-terminal PDZ ligand sequences.

Spinophilin is also known to associate with protein phosphatase-1. PP-1 is a ubiquitously expressed protein phosphatase; its activity is regulated in different tissues by tissue-specific binding proteins (24). In the nervous system, cells that receive dopaminergic input contain DARPP-32 (dopamine and cAMP regulated phosphoprotein, M_r = 32,000), which, when phosphorylated in response to elevations in cAMP and protein kinase A activity mediated by D1 receptor activation, is a potent inhibitor of PP-1 activity. Recent data show that D2-selective agonists negatively regulate phosphorylation of DARPP-32 (25). In the initial description of spinophilin, the site of interaction with PP-1 was not mapped (10). Several groups have reported consensus motifs for anchoring/targeting protein interactions with PP-1 (26, 27); spinophilin contains such a motif between amino acids 447–451 (R-K-I-H-F-S). We tested whether this site could mediate the interaction with PP-1 by incubating ^{35}S-labeled PP-1γ1 with the various GST-spinophilin fusion proteins as above. Fig. 3*C* shows that PP-1γ1 bound specifically to all GST-spinophilin fusion proteins containing the putative PP-1
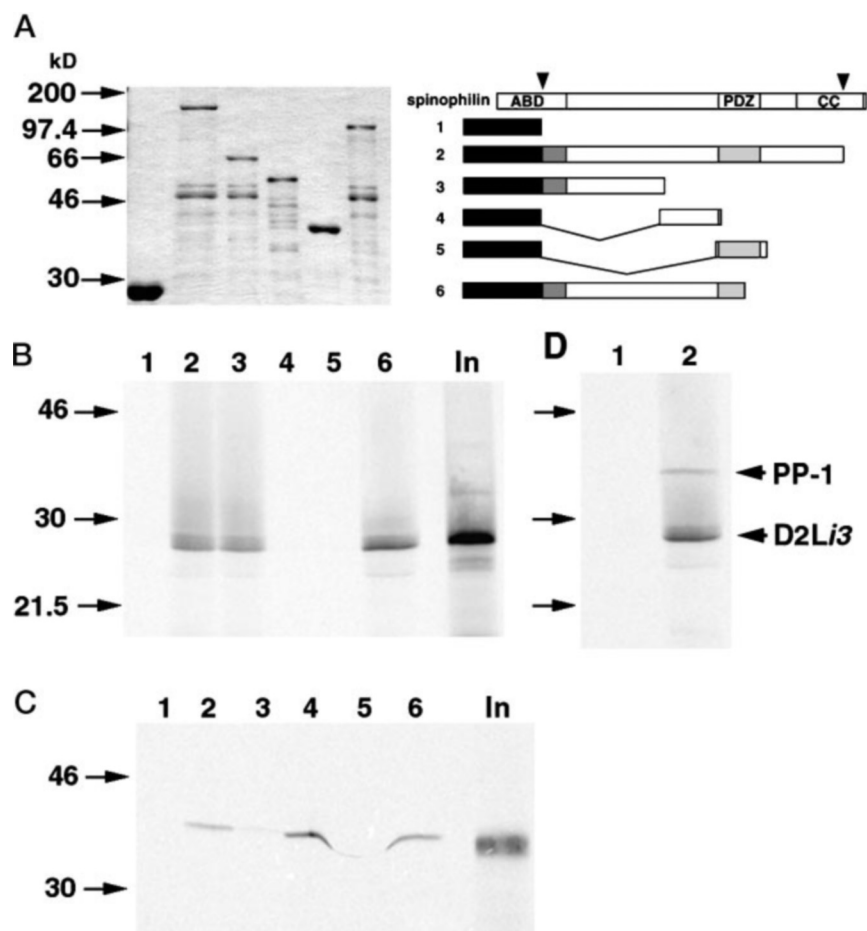
FIG. 3. **Mapping the interactions between spinophilin, D2 receptors, and PP-1.** A series of GST fusion proteins shown schematically (*A, right*) were expressed and purified from bacteria as shown in the Coomassie-stained gel (*A, left*). The fusions proteins are as follows: *lane 1*, GST alone; *lane 2*, GST-spinophilin (100–767) (two-hybrid clone, as marked by arrowheads); *lane 3*, GST-spinophilin (100–371); *lane 4*, GST-spinophilin (354–494); *lane 5*, GST-spinophilin (488–593); *lane 6*, GST-spinophilin (100–543). *B*, 5 μl of *in vitro* translated ^{35}S-labeled His$_6$-D2L*i3* were incubated with each GST fusion protein immobilized on beads. Bound proteins were visualized by SDS-polyacrylamide gel electrophoresis and a PhosphorImager analysis. One-half the input to each reaction was analyzed (*In*). The recombinant D2L third cytoplasmic loop bound specifically to GST-spinophilin fusion proteins containing amino acids 100–371. *C*, because spinophilin is known to bind PP-1, we tested where this binding occurs using the set of GST-spinophilin fusion proteins and *in vitro* translated PP-1γ1. PP-1 binds to spinophilin in the region between amino acids 371–488, at a site distinct from the D2L*i3*-binding site, because GST-spinophilin (100–371) can still bind D2L*i3* but not PP-1, and GST-spinophilin (354–494) can bind PP-1 but not D2L*i3*. Therefore, D2 receptors and PP-1 may interact with spinophilin at distinct sites at the same time, as is shown in *D*. Lane numbers in *B*, *C*, and *D* correspond to the fusion proteins diagramed in *A*.

interaction site. Blot overlay assays with purified, digoxygenin-labeled PP-1 confirm that these three GST fusion proteins alone are able to bind PP-1.[2] Binding of PP-1 was lost when spinophilin was truncated to delete this motif (Fig. 3C, *lane 3*, GST-spinophilin (100–371)). Interestingly, the same fragment was still able to bind to the third cytoplasmic loop of D2L (Fig. 3B). These data indicate that D2 dopamine receptors bind to spinophilin through a distinct region upstream of the PP-1-binding site (Fig. 3). Furthermore, we tested whether D2L*i3* and PP-1 could associate with spinophilin at the same time. Both radiolabeled proteins were able to bind GST-spinophilin when tested in the same reaction (Fig. 3*D*). Although this result does not prove that the D2 third cytoplasmic loop and PP-1 interact with the same spinophilin molecule, it is consistent with the characterization of spinophilin as a linker protein that brings intracellular signaling molecules (PP-1) into close proximity of a membrane bound receptor (D2).

Our data suggest that the interaction between the third cytoplasmic loop of D2 receptors and spinophilin occurs within the amino-terminal part of the region in spinophilin bridging the actin-binding domain and the PDZ domain. This segment is

not well conserved among known proteins. In fact, spinophilin is similar to only one other protein, neurabin, in overall structural organization and primary sequence (11, 16).

Alignment of the corresponding regions of spinophilin (100–371) and neurabin (101–372) reveals limited sequence identity, and ~35% sequence similarity (Fig. 4*A*). We were therefore interested in whether both spinophilin and neurabin could bind to the D2L*i3* loop. The regions of spinophilin and neurabin above were produced as GST fusion proteins and assayed for binding to ^{35}S-labeled D2L*i3* loop. GST-spinophilin (101–370) specifically bound to the D2 third cytoplasmic loop (Fig. 4*B*, *lane 2*), whereas the corresponding region of neurabin did not bind to the receptor loop (Fig. 4*B*, *lane 3*). These data suggest that neurabin and spinophilin may serve distinct functions *in vivo* and furthers the suggestion of overlapping but distinct sets of binding partners. However, we have not tested other domains in neurabin and cannot rule out that another site in neurabin is responsible for binding the third cytoplasmic loop of D2 (or possibly other GPCRs).

The functional significance of the two splice variants of D2 is still not understood. We therefore tested whether or not the interaction with spinophilin is specific for D2L *versus* D2S using both the yeast two-hybrid system and *in vitro* binding
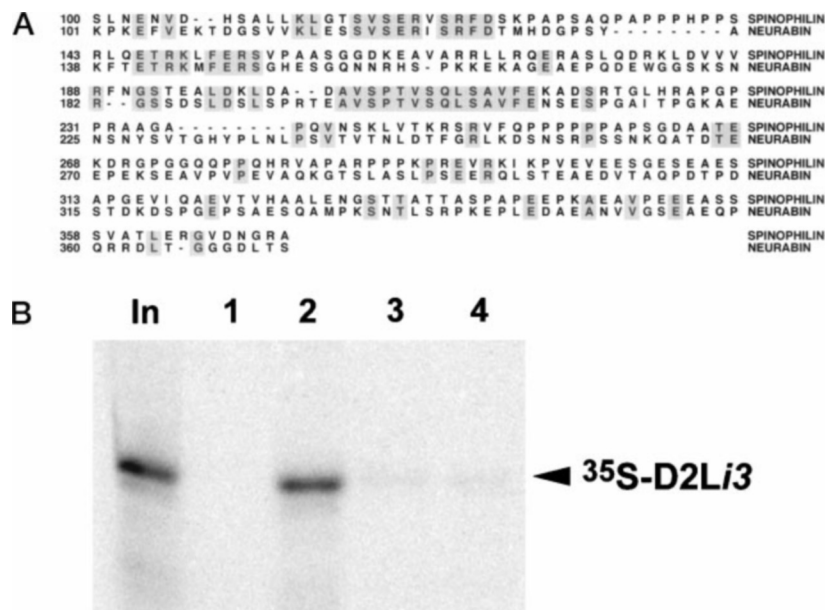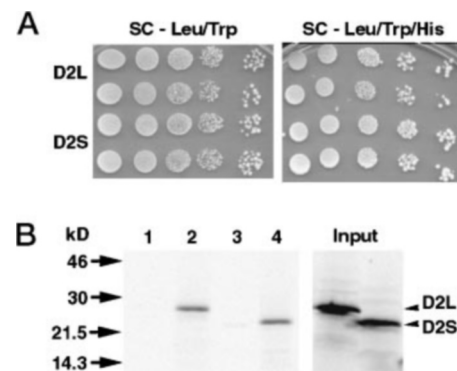
FIG. 4. **The D2 third cytoplasmic loop specifically interacts with spinophilin but not its closest relative, neurabin.** *A*, alignment of the region of spinophilin that binds to D2L*i3*, amino acids 100–371, with the corresponding region of the related protein neurabin (amino acids 101–372), reveals low sequence similarity. There are, however, several clusters of high homology in this region. *B*, the portions of spinophilin and neurabin listed above were expressed as GST fusion proteins and tested for interaction with ^{35}S-labeled D2L*i3*. Only spinophilin was able to bind to the labeled D2 receptor loop. *Lane In*, input; *lane 1*, GST; *lane 2*, GST-spinophilin (100–371); *lane 3*, GST-neurabin (101–372); *lane 4*, GST-spinophilin (354–494).

assays as described above. The third cytoplasmic loops of both D2L and D2S exhibit a comparable interaction; both are able to activate transcription of reporter genes in yeast and both bind specifically to GST-spinophilin fusion proteins when expressed *in vitro* as ^{35}S-labeled proteins (Fig. 5). These data suggest that the 29-amino acid insert that is unique to the third cytoplasmic loop of D2L is not responsible for the interaction with spinophilin and that functional differences between the long and short isoforms are unlikely to arise from differential interactions with spinophilin.

Although the data above demonstrate that the interaction between spinophilin and the third cytoplasmic loop of D2L and D2S seen in the yeast two-hybrid system also occurs as a direct interaction *in vitro*, they do not address the issue of an *in vivo* interaction. To begin to study this question, we constructed and expressed an HA-tagged D2S receptor in MDCK cells. MDCK cells are a cultured kidney epithelial cell line that grow as confluent monolayers of polarized cells. Previously, Satoh *et al.* (11) showed that spinophilin is endogenously expressed in MDCK cells and is localized to the lateral cell surface, overlapping markers for adherens junctions. We expressed the HA-tagged receptor in these cells to see whether it would be targeted to the same subcellular domain as spinophilin. Saturation binding assays with the radioligand [^{3}H]spiperone indicate the presence of functional ligand-binding sites only in the cells stably transfected with the HA-D2S construct (data not shown). Spinophilin was detected in these cells using rabbit polyclonal antibody NC152, and the receptor was detected using antibodies against the HA tag (BabCo). Spinophilin is localized to the lateral surface of MDCK cells (Fig. 6*A*), as reported by Satoh *et al.* (11). However, we also detect spinophilin staining apically in cells grown on Transwell filters, as well as a considerable amount of punctate intracellular staining (Fig. 6*B*). HA-D2S dopamine receptors are targeted to the lateral surface in these cells (Fig. 6), although as the expression level increases, significant amounts of receptor seem to be produced and stored in intracellular pools (data not shown). Merged images of the staining patterns of spinophilin and HA-D2S through a single optical slice indicate that the subcellular lo-

FIG. 5. **The interaction between both long and short splice variants of the D2 third cytoplasmic loops with spinophilin was tested in both yeast two-hybrid and *in vitro* binding assays.** *A*, the interaction between D2L*i3* or D2S*i3* and spinophilin conferred a strong His$^+$ phenotype in the two-hybrid assay. *B*, *in vitro* translated ^{35}S-labeled D2L*i3* and D2S*i3* bound specifically to GST-spinophilin (100–767) (*lanes 2* and *4*) but not to GST alone (*lanes 1* and *3*).

calization of these two proteins overlaps significantly, although not completely (data not shown), providing additional evidence for the interaction described above. Although these data do not provide direct evidence for an *in vivo* interaction, they do suggest that spinophilin and D2 receptors are found in the same membrane domain in polarized cells and that when co-expressed, they will be well positioned to form the interaction we observe *in vitro*. Currently, we are unable to co-immunoprecipitate a D2 receptor-spinophilin complex. We speculate that this interaction may be intricately regulated *in vivo* and are exploring these possibilities. It will be important in the future to determine whether the disruption of the spinophilin-D2 receptor interaction has significant effects on downstream signaling events controlled by these receptors.

Although we have co-localized spinophilin and D2 receptors in MDCK cells, it has yet to be determined whether these two molecules associate in neurons. One current hypothesis suggests that the polarity of membrane domains of epithelial cells
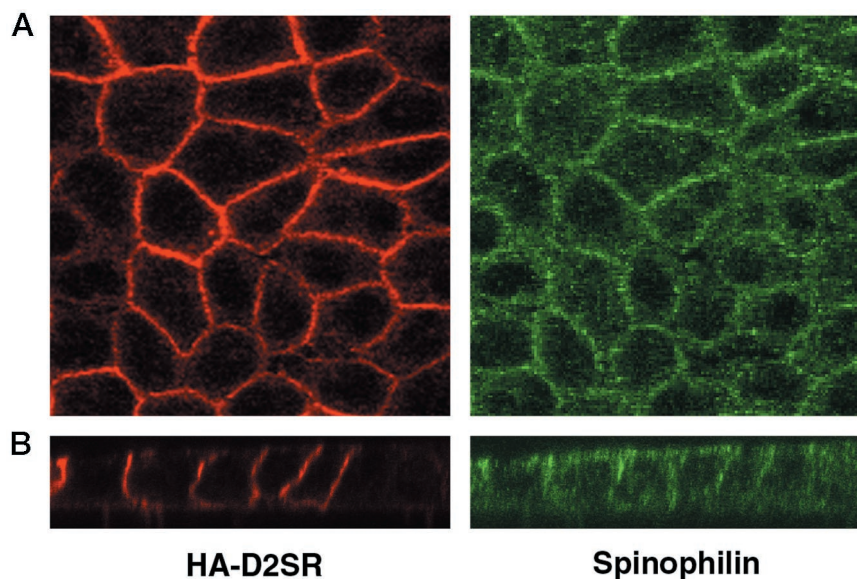
FIG. 6. **Spinophilin and D2 receptors are targeted to the same membrane domain in MDCK cells.** MDCK cells endogenously expressing spinophilin were stably transfected with pRc/RSV-D2S-HA, expressing an extracellularly HA-tagged D2S receptor. Cells were grown on Transwell filters until forming tight monolayers (5–7 days) and fixed in −20 °C methanol. Immunolocalization of spinophilin and HA-D2S was done using rabbit α-spinophilin (1:100) and mouse α-HA (1:500) primary antibodies and visualized using α-rabbit or α-mouse 2° antibodies coupled to Oregon Green or Texas Red, respectively. *A*, *xy* sections showing HA-D2R and spinophilin staining localized to cell-cell junctions along the lateral surface. Spinophilin staining appears more punctate and diffuse within the cell. Because of the nonclonal nature of the HA-D2R cell line, levels of receptor expression vary among cells in the field. *B*, *xz* sections show that although the D2 receptor is strictly lateral, spinophilin is found laterally and subapically in many cells. Additionally, some spinophilin appears to be associated with diffuse puncta within the cytoplasm. However, there is clear overlap between the populations of D2 receptor and spinophilin, placing these proteins in close proximity.

mimics that of neurons (28, 29). It is not clear that such a model holds true in all cases (30). Therefore, it is necessary to look for an association between D2 receptors and spinophilin in neurons and to begin to probe the functional consequences of this interaction.

Spinophilin and neurabin seem to be defining members of a family of single PDZ domain proteins that serve as direct links to the actin cytoskeleton. These two proteins share little homology with known proteins outside of the domains mentioned above but are themselves between 41 and 86% identical (11). The highest degree of homology is in the PDZ domain and in the region adjacent to and including the coiled-coil(s). These similarities again suggest that the two proteins may have overlapping but distinct functions *in vivo*. Recently, p70^{S6} kinase was shown to interact with the PDZ domain of neurabin through a carboxyl-terminal LRMNL$_{COOH}$ motif (31). This kinase is responsible for the phosphorylation of the S6 protein component of the 40 S subunit of ribosomes (32). Phosphorylation generally occurs in response to mitogenic signals and results in an increased rate of protein synthesis (31, 32). Spinophilin may provide a link between D2 dopamine receptors and intracellular mitogenic signaling events dependent on p70^{S6} kinase. Indeed, in Chinese hamster ovary cells stably expressing D2 receptors, dopamine application elicits a wortmannin and rapamycin-sensitive activation of p70^{S6} kinase (33). This activation is pertussis toxin-sensitive, suggesting that dopamine stimulation of p70^{S6} kinase activity proceeds through G$_{i/o}$ class G proteins (33).

Alternatively, spinophilin may serve as a scaffolding/adapter protein for multiple GPCRs. It is possible that spinophilin plays a central role in coordinating signaling pathways mediated by several different G$_i$-coupled receptors instead of acting specifically at a single type of receptor. Thus, a careful examination of the interactions between spinophilin and various membrane receptors and cytoplasmic signaling molecules will greatly aid our understanding of the specificity and diversity of GPCR signaling.

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