A growing number of proteins containing PDZ domains have been shown to play important roles in the organization and/or regulation of signaling events in cells. PDZ domains (or GLGF repeats) were named after three proteins identified over a decade ago: postsynaptic density-95, Drosophila Discs large, and zonula occludens-1 (3–5). These three proteins belong to the membrane-associated guanylate kinase (MAGUK) family of proteins. Most MAGUK proteins contain three PDZ domains, an Src homology 2 domain, and a guanylate kinase-like domain, each having different cellular roles. PDZ domains range from 80 to 100 amino acids in length and typically bind to the carboxyl-terminal sequence of target proteins including receptors, channels, and various signaling molecules to regulate subcellular localization, trafficking, recycling, and/or signaling (6–10).

MUPP1, a protein containing 13 putative PDZ domains, was isolated in a yeast two-hybrid screening for proteins that bound to the carboxy-terminal tail of the 5-HT$_{2C}$ R (1). MUPP1 is expressed in many tissues, whereas the 5-HT$_{2C}$ R is a brain-specific protein (1, 11). The 5-HT$_{2C}$ R has classically been thought to couple to G$_q$ activation; however, additional G protein families have been implicated, leading to the activation of different downstream signaling pathways including phospholipase A$_2$, C, or D, and various cation channels (12–17). Since PDZ-containing proteins can scaffold many signaling molecules together into a signal transduction complex, the interaction between MUPP1 and the 5-HT$_{2C}$ R was further investigated. The 5-HT$_{2C}$ R contains a PDZ binding motif, Ser$_{458}$Ser-Val, at its extreme carboxyl terminus, which is critical for interaction with PDZ 10 of MUPP1 (18). In an alternate approach to the yeast two-hybrid system, we independently show that PDZ 10 of MUPP1 is the primary site of interaction for the 5-HT$_{2C}$ R. Serotonin stimulation has previously been shown to promote phosphorylation of the two serine residues of the 5-HT$_{2C}$ R PDZ binding motif, Ser$_{458}$ and Ser$_{459}$ (2). We therefore hypothesize that phosphorylation of the carboxyl-terminal serines of the 5-HT$_{2C}$ R regulates receptor interaction with MUPP1. To test this hypothesis, we investigated whether a modification of Ser$_{458}$ and/or Ser$_{459}$ of the 5-HT$_{2C}$ R carboxy-terminal tail would alter PDZ 10 interaction. Ser$_{458}$ and/or Ser$_{459}$ of the receptor tail were mutated to aspartate to mimic phosphorylation (i.e. introduction of a negative charge). Next, cells expressing 5-HT$_{2C}$ Rs were treated with agonist or antagonist to assess the interaction of the 5-HT$_{2C}$ R with MUPP1. The results of these experiments support our hypothesis that phosphorylation is a key regulator of 5-HT$_{2C}$ R interaction with MUPP1. Furthermore, the results indicated that a significant amount of basal phosphorylation of the receptor may also play a yet undetermined role in regulating PDZ-protein interactions.

MATERIALS AND METHODS

Antibodies

Polyclonal anti-peptide antibodies against amino acids 419–435 (amino acids RHTNERVARKANDPEPG) of the rat 5-HT$_{2C}$ R were generated as described previously (19). Anti-glutathione S-transferase (GST) antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

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Phosphorylation Regulates PDZ-5-HT$_{2C}$ Receptor Interaction

DNA Constructs

Overlapping regions of MUPP1 containing two or three PDZ domains (Fig. 1), or one PDZ domain (PDZ 9, 10, or 11) were generated by reverse transcription-PCR, sequenced, and subcloned into pGEX-4T1 (Amer- sham Biosciences) for expression of GST fusion proteins. MUPP1 PDZ 9, 10, or 11 were also subcloned into pGEMEX-1 (Promega), a T7 gene 10 fusion protein vector. MUPP1 PDZ 9–11 was also subcloned into pcDNA3 (Invitrogen). The 5-HT$_{2C}$ R carboxyl-terminal tail (last 60 amino acids) with or without the PDZ binding motif (Ser$_{445}$-Ser-Val) and a truncation mutant at residue 445 were subcloned into pGEX-1. The 5-HT$_{2C}$ R carboxyl-terminal tail with the PDZ binding motif was also subcloned into pGEX-4T1. The 5-HT$_{2C}$ R carboxy tail Ser$_{445}$-Ser-Val (WT) was modified to S458A, S458D, S459D, or S458D/S459D by PCR site-directed mutagenesis and subcloned into pGEMEX-1.

GST Fusion Protein Overexpression

Escherichia coli was transformed with pGEX-4T1 constructs, in- ducted to overexpress fusion proteins with isopropyl $\beta$-thiogalactoside, and analyzed. Bacterial lysates were obtained by first adding cold lysis buffer (50 mM Tris pH 7.5, 50 mM NaCl, 5 mM MgCl$_2$, 1 mM dithio- rotol, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml papain A, 1 $\mu$g/ml aprotinin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride) to resuspend the pellets. Resuspended pellets were sonicated for 20 s on ice and centrifuged at 15,000 rpm for 30 min at 4°C. Proteins were resolved on SDS-PAGE to confirm overexpressed GST fusion protein by Coomassie Blue staining and Western blotting using GST antibodies.

Protein Overlay Assays

Ten micrograms of GST fusion proteins or GST were size-fraction- ated on SDS-PAGE and transferred onto nitrocellulose membranes for autoradiography or PhosphorImager screen was used to visualize radiolabeled proteins. Western blot analysis using GST and GST 5-HT$_{2C}$ R antibodies was used to document GST fusion proteins and lose membranes. Autoradiography or PhosphorImager screen was used to visualize radiolabeled proteins. Western blot analysis using GST and GST 5-HT$_{2C}$ R antibodies were used to document GST fusion proteins and 5-HT$_{2C}$ R, respectively.

GST Fusion Protein Overexpression

Escherichia coli was transformed with pGEX-4T1 constructs, in- ducted to overexpress fusion proteins with isopropyl $\beta$-thiogalactoside, and analyzed. Bacterial lysates were obtained by first adding cold lysis buffer (50 mM Tris pH 7.5, 50 mM NaCl, 5 mM MgCl$_2$, 1 mM dithio- rotol, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml papain A, 1 $\mu$g/ml aprotinin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride) to resuspend the pellets. Resuspended pellets were sonicated for 20 s on ice and centrifuged at 15,000 rpm for 30 min at 4°C. Proteins were resolved on SDS-PAGE to confirm overexpressed GST fusion protein by Coomassie Blue staining and Western blotting using GST antibodies.

Protein Overlay Assays

Ten micrograms of GST fusion proteins or GST were size-fraction- ated on SDS-PAGE and transferred onto nitrocellulose membrane. Ni- trocellulose membranes were blocked with freshly prepared 1% BSA/ phosphate-buffered saline for 1 h at room temperature. Solution was then replaced with $^{35}$S-labeled fusion proteins in 1% BSA/phosphate- buffered saline buffer and incubated with nitrocellulose membranes for 16 h at 4°C. Nitrocellulose membranes were rinsed three times for 20 min at room temperature in 1% BSA/phosphate-buffered saline contain- ing 0.2% Triton X-100. Nitrocellulose membranes were air-dried and exposed to x-ray film or a PhosphorImager screen (Amer sham Biosciences) to visualize radiolabeled proteins. Western blot analysis using GST antibodies was used to document similar GST protein levels.

Cell Culture

NIH-3T3 cells stably transfected with the 5-HT$_{2C}$ R (5-HT$_{2C}$ R/3T3) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) until confluent (20). Cells were washed four times with Hank’s buffered saline solution (with Ca$^{2+}$/Mg$^{2+}$) and then serum starved in serum-free Dulbecco’s modified Eagle’s medium for 16 h. Cells were treated with- out or with antagonist (1 $\mu$m 2-bromo-lysergic acid diethylamide (BOL)) for 15 min at 37°C prior to serotonin addition for 30 min at 37°C. Medium was then removed, 1 ml of Tris buffer (50 mM Tris, pH 7.6, 0.5 mM EDTA, pH 8.0, 1 $\mu$m leupeptin, 1 $\mu$m phenylmethylsulfonyl fluoride) was added, and cells were scraped from plates and placed in an Eppen-dorf tube on ice. Membrane extracts were obtained using 300 $\mu$l of Tris buffer containing 1% Triton X-100. Membrane protein concentrations were determined by BCA protein assay (Pierce). Equal amounts of protein were added to the affinity columns. Western blot analysis using GST antibodies was used to determine that similar levels of fusion protein were pulled down.

Pulldown Assays

Twenty microliters of glutathione-Sepharose beads (Amer sham Biosciences) were washed three times with PD buffer (20 mM HEPES, pH 7.6, 100 mM KCl, 10% glycerol, 0.5 mM EDTA, pH 8.0, 1 mM phenyl- methylsulfonyl fluoride, 1 mM dithirotol, 1% Nonidet P-40). Ten micrograms of GST fusion proteins were added to the washed glutathione beads for 1 h at 4°C. Five microliters of $^{35}$S-labeled fusion proteins or 50 $\mu$g of membrane extracts were added to the GST-glutathione beads and incubated for 2–3 h or overnight, respectively, at 4°C. After incubation, GST-glutathione beads were washed six times (for assays with $^{35}$S-labeled fusion proteins) or three times (for assays with membrane extracts) with PD buffer. For pull-downs from NIH-3T3 cell lysates, precipitated protein, containing the 5-HT$_{2C}$ R, was treated with peptide-$N$-glycanase F before SDS-PAGE (see below). Loading dye (6% SDS, 1% $\beta$-mercaptoethanol, 20 mM Tris, pH 6.8, 10% glycerol plus a little bromphenol blue) was added to elute proteins. Eluates were separated on SDS-PAGE and transferred onto nitrocellulose membranes. Autoradiography or PhosphorImager screen was used to visualize radiolabeled proteins. Western blot analysis using GST and 5-HT$_{2C}$ R antibodies were used to document GST fusion proteins and 5-HT$_{2C}$ R, respectively.

Band Shift Phosphorylation Assay

This assay was performed as previously described by Backstrom et al. (2). Briefly, cells grown to confluence were serum-starved and treated with increasing amounts of serotonin for 15 min at 37°C. This incubation time was previously shown to be the minimal amount of time that would result in maximal receptor phosphorylation. Cells were then lysed, and membrane extracts containing receptors were prepared. Western blot analysis using 5-HT$_{2C}$ R antibodies were used to document phospho-MUPP1 PDZ 9 sites in 40- and 41-kDa bands, representative of unphosphorylated and phosphorylated 5-HT$_{2C}$ R, respectively.

Deglycosylation

Following pull-downs from membrane extracts, beads were pelleted and washed once with PD buffer containing 0.1% SDS. Fifteen micro- liters of PD buffer containing 1% SDS was added to the beads, and the mixture was incubated for 15 min at 37°C. Then 58 $\mu$l of PD buffer was added, and after mixing, 15 $\mu$l of PD buffer containing 10% Triton X-100 was added. Finally, 2 $\mu$l of peptide-$N$-glycanase F (Glyko or New Eng- land Biolabs) was added, and samples were incubated for 2 h at 37°C. After deglycosylation, 15 $\mu$l of 4× loading dye was added, and samples were incubated at room temperature for 20 min prior to SDS-PAGE.

Dephosphorylation

Membrane extracts (50 $\mu$g) of untreated or serotonin-treated cells were incubated in PD buffer plus 50 units of calf intestinal (alkaline) phosphatase (New England Biolabs) for 2 h at room temperature. After incubation, pull-down assays were carried out as described above.

Western Blot Analysis

Alkaline Phosphatase Detection—Nitrocellulose membranes were blocked in 1% BSA/Tris blot buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 0.05% Na$_3$PO$_4$) for 1 h at room temperature. Membranes were then incubated with GST (1:1000 dilution) or 5-HT$_{2C}$ R (3–5 $\mu$g/ml antibodies in 1% BSA/Tris blot buffer for 2 h to overnight at 4°C. Membranes were washed three times with Tris blot buffer alone for 10 min. Alkaline phosphatase-conjugated goat anti-rabbit secondary anti- bodies (1:1000 dilution; Jackson Immunolaboratories) were incubated with membranes for 2 h at room temperature. Membranes were washed three times with Tris blot buffer and once with Tris (150 mM pH 7.4) and then developed with 5-bromo-4-chloro-3-indolyl-phosphate and ni- tro blue tetrazolium.

Chemilluminence Detection—Nitrocellulose membranes were blocked in 8% milk/Tween 20 Tris buffer solution (25 mM Tris, pH 7.4, 137 mM NaCl, 0.27 mM KCl, 0.05% Tween 20) overnight at 4°C. Mem- branes were then incubated with GST (1:4000 dilution) or 5-HT$_{2C}$ R (0.5 $\mu$g/ml antibodies in 2% milk/Tween 20 Tris buffer solution overnight at 4°C. Membranes were washed four times for 5 min with TWEEN 20 Tris buffer solution. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:20,000 dilution; Jackson Immunolaboratories) were incubated with membranes for 45 min at room temperature. Membranes were washed four times for 15 min with TWEEN 20 Tris buffer solution and developed with the Pierce SupernSignal West Dura® kit according to the supplier’s protocol. Horseradish peroxidase signal was analyzed by Bio-Rad Flouro-S, and densitometric analysis was performed by QuantityOne (Bio-Rad) software.
**Statistical Analysis**

All bar graph data was analyzed with Graphpad Prism one-way analysis of variance with Tukey’s post-test; *p* < 0.05 is significant, unless otherwise noted in a figure legend. GST alone was the background control for all GST fusion protein experiments, and graph data presented are background-subtracted. Data represent the means ± S.D. from several independent experiments.

**RESULTS**

The 5-HT$_{2C}$R Receptor Selectively Interacts with MUPP1 PDZ 10—The carboxyl region of MUPP1 containing the last four PDZ domains (PDZ 10 to PDZ 13) was originally identified by yeast two-hybrid screening for 5-HT$_{2C}$R-interacting proteins (1). However, it was unclear which PDZ domain interacted with the 5-HT$_{2C}$R. Therefore, we set out to identify which domain(s) of MUPP1 interacts with the 5-HT$_{2C}$R. Overlapping PDZ domain regions of MUPP1 were generated as GST fusion proteins (Fig. 1). Purified GST-MUPP1 PDZ domains were used to pull-down in vitro translated $^{35}$S-labeled 5-HT$_{2C}$R carboxyl terminus fusion protein, which consists of the last 60 amino acids harboring a PDZ binding motif, Ser$^{458}$-Ser-Val. Fig. 2A illustrates that significantly more receptor tail interacted with PDZ 9–11 and PDZ 9–13. A weak interaction of the receptor tail over GST alone was observed with PDZ 12 and 13, suggesting that PDZ 9–11 is the primary 5-HT$_{2C}$R interacting region. In protein overlay assays, GST-MUPP1 PDZ domain fusion proteins were blotted and probed with $^{35}$S-labeled 5-HT$_{2C}$R carboxyl terminus, and the 5-HT$_{2C}$R tail specifically interacted with PDZ 9–11 and PDZ 9–13; no other PDZ domains displayed a significant interaction with the receptor tail (results not shown). Thus, both protein overlay and pull-down assays consistently indicate that PDZ 9–11 is responsible for interacting with the 5-HT$_{2C}$R tail.

To further determine the specific site of interaction, GST fusion proteins of the individual PDZ domains, 9, 10, and 11 were made (Fig. 1). Unfortunately, GST-PDZ 10 was unstable when overexpressed in bacteria. Therefore, the ability to pull down the $^{35}$S-labeled 5-HT$_{2C}$R tail by GST-PDZ 9 or 11 was compared with GST-PDZ 9–11. GST fusion proteins of PDZ 9 or 11 alone were not able to bind to the receptor tail as compared with GST-PDZ 9–11, which contains PDZ 10 (data not shown). In a complementary pull-down experiment, the individual MUPP1 PDZ domains 9, 10, and 11 were in vitro translated with $^{35}$Smethionine and pulled down by the 5-HT$_{2C}$R carboxyl-terminal tail expressed as a GST fusion protein (Fig. 2B). The carboxyl tail of the receptor specifically interacted with the 5-HT$_{2C}$R.

### Fig. 1

**Schematic diagram of MUPP1 PDZ domains.** MUPP1 has 2054 residues. Each numbered block (grey) represents the 13 individual PDZ domains of MUPP1. Pairs or individual PDZ domains were generated as GST or T7 gene 10 fusion proteins to test for 5-HT$_{2C}$R interaction. Numbers under each block denote amino acid range.

### Fig. 2

**MUPP1 PDZ 10 interacts with the carboxyl terminus of the 5-HT$_{2C}$R.** Pull-down assays were used to determine which MUPP1 PDZ domain(s) interacted with the receptor. A, GST-MUPP1 PDZ domains bound to glutathione beads were incubated with $^{35}$S$^{35}$S-5-HT$_{2C}$R R carboxyl tail. Radioactive fusion receptor tail that bound to specific PDZ domains was resolved by SDS-PAGE, transferred onto nitrocellulose, and analyzed by autoradiography or PhosphorImager. Both PDZ 9–11 and PDZ 9–13 bound significantly more radiolabeled receptor than other PDZ domains. Similar amounts of fusion proteins were used as demonstrated by Western blotting (not shown). The bar graph represents *n* = 6; *, *p* < 0.001 relative to GST. The panel above the graph is a representative pull-down result. B, the last 60 amino acids of the 5-HT$_{2C}$R were used to pull down $^{35}$S$^{35}$S-MUPP1 PDZ domain 9, 10, or 11. Radioactive PDZ domain pulled down was analyzed by autoradiography or PhosphorImager. Top panel, PDZ 10 was specifically pulled down by the 5-HT$_{2C}$R carboxyl tail fusion but not by GST alone. Equal amounts of GST fusion or GST proteins were shown by Western blotting (*n* = 2; bottom panel).
with PDZ 10 and not PDZ domain 9 or 11, further supporting PDZ 10 as the interacting region for the receptor tail.

Next, we questioned whether regions upstream of the extreme carboxyl terminal of the 5-HT$_{2C}$R are able to confer binding to PDZ 10. To address this question, we generated GST fusion proteins of the 5-HT$_{2C}$R carboxyl-terminal tail missing the last 15 amino acids (i.e. terminal tail ending at residue 445). In an overlay assay, [35S]PDZ 9–11 was incubated with nitrocellulose blots, and then blots were exposed to film after the removal of nonspecific binding. MUPP1 PDZ 9–11 positively interacted with WT 5-HT$_{2C}$R carboxyl tail, whereas ΔPDZ and 445 did not exhibit any detectable binding (top panel). A nonspecific band was observed that is also present in the GST alone lane. Western blotting was used to show the presence of fusion protein (n = 3) (bottom panel).

Fig. 3. The terminal residues of the 5-HT$_{2C}$R are necessary for PDZ 10 interaction. Protein overlay assay was employed to assess what region of the carboxyl-terminal tail of the 5-HT$_{2C}$R is needed for binding to MUPP1. GST fusion proteins of the 5-HT$_{2C}$R carboxyl tail with the PDZ binding motif (WT), without the PDZ motif (Δ PDZ), and a truncated mutant at amino acid 445 (445) were separated by SDS-PAGE and transferred onto nitrocellulose blots. [35S]MUPP1 PDZ 9–11 was incubated with nitrocellulose blots, and then blots were exposed to film after the removal of nonspecific binding. MUPP1 PDZ 9–11 positively interacted with WT 5-HT$_{2C}$R carboxyl tail, whereas ΔPDZ and 445 did not exhibit any detectable binding (top panel). A nonspecific band was observed that is also present in the GST alone lane. Western blotting was used to show the presence of fusion protein (n = 3) (bottom panel).

Mutation of Ser$_{458}$ in the 5-HT$_{2C}$R Receptor Reveals Altered PDZ 10 Interaction—Studies previously demonstrated that Ser$_{458}$ and Ser$_{459}$ at the extreme carboxyl tail of the 5-HT$_{2C}$R, the same region of the receptor necessary for PDZ 10 binding, are phosphorylated upon ligand activation (2). A function for Ser$_{458}$ phosphorylation in receptor resensitization was proposed; however, the role for Ser$_{458}$ phosphorylation is unknown. Based upon crystal structures of PDZ domains (21–25) and data compiled on PDZ binding motifs (26), Ser$_{458}$ of the 5-HT$_{2C}$R is predicted to be a critical residue for interacting with PDZ 10. We therefore hypothesized that agonist-mediated phosphorylation of Ser$_{458}$ disrupts the interaction of MUPP1 PDZ domain 10 and its target, the 5-HT$_{2C}$R. To test this hypothesis, the serine residues in the receptor tail were replaced with aspartic acid to mimic phosphorylation. The last two serine residues of the 5-HT$_{2C}$R carboxyl tail (Ser$_{458}$-Ser$_{459}$) were modified by PCR site-directed mutagenesis to contain S458A, S458D, S459D, or S458D/S459D substitutions. Wild-type and mutated 5-HT$_{2C}$R tails were labeled with [35S]methionine and incubated with GST-PDZ 9–11. 5-HT$_{2C}$R tail mutants containing S458A, S458D, and S458D/S459D substitutions displayed a marked loss of interaction to PDZ 9–11 (Fig. 4A). The S459D mutation, however, retained an ability to interact similar to wild-type interaction (Fig. 4B). These results indicate that Ser$_{458}$ is an important residue in determining the interaction with PDZ 10.

Serotonin Treatment Decreases the Ability of the 5-HT$_{2C}$R to Interact with PDZ 10—Results from the 5-HT$_{2C}$R tail mutants raise the possibility of a dynamic regulation of the interaction between the 5-HT$_{2C}$R and MUPP1. Thus, we investigated whether agonist stimulation of the 5-HT$_{2C}$R stably expressed in NIH-3T3 cells would also result in a loss of MUPP1 interaction. To determine whether serotonin stimulation had any effect on MUPP1-receptor interaction, cells were incubated with increasing amounts of serotonin, which have been shown to promote receptor phosphorylation (2). The ability of the 5-HT$_{2C}$R to bind to PDZ 10 was assessed by pull-down assays. Fig. 5A shows that cells treated with serotonin led to a dose-dependent decrease in receptor interaction with MUPP1. A 50% reduction in receptor binding to PDZ 10 was observed with a concentration 100 nM serotonin. Moreover,
increasing serotonin concentrations caused a dose-dependent increase in phosphorylated receptor with a concurrent decrease in the amount of unphosphorylated receptor as determined by band shift phosphorylation assays (Fig. 5B).

To determine whether the loss of PDZ 10 interaction with the receptor was a consequence of agonist binding with 5-HT$_{2C}$ Rs, cells were preincubated in the absence or presence 1 µM of BOL, a 5-HT$_{2C}$ R antagonist, for 15 min prior to the addition of serotonin. BOL antagonized a subsequent serotonin-mediated decrease in receptor pull-down (Fig. 6), thereby demonstrating that the loss of PDZ 10 interaction is a direct consequence of receptor activation. BOL alone had no effect.

Alkaline Phosphatase Treatment of the 5-HT$_{2C}$ R decreases 5-HT$_{2C}$ R binding to PDZ 10 and Reveals 5-HT$_{2C}$ R Basal Phosphorylation—The reduction of 5-HT$_{2C}$ R binding to MUPP1 may be the direct result of receptor phosphorylation. We therefore investigated whether treatment of lysate containing receptor with alkaline phosphatase would restore MUPP1 interaction. Cells were treated with agonist, and cell lysates were incubated with alkaline phosphatase prior to pull-down assays. As shown in Fig. 7A, alkaline phosphatase treatment resulted in more receptor pull-down in serotonin-stimulated cells. In the absence of serotonin, alkaline phosphatase treatment doubled the amount of receptor binding to PDZ 10 compared with untreated cells. These findings directly support a role for agonist-induced phosphorylation in disrupting 5-HT$_{2C}$ R binding to MUPP1 as well as uncover a potential function for previously reported basal phosphorylation of the receptor.

Phosphorylation of the receptor is reversible; therefore, we investigated the activity of endogenous phosphatases against the receptor by washout experiments. Cells were treated with agonist and then washed thoroughly and incubated in serum-free medium for 10 or 30 min before lysis and pull-down assay. Fig. 7B demonstrates a time-dependent increase in 5-HT$_{2C}$ R binding to MUPP1 (Fig. 7B). These results are consistent with previously published data indicating a time-dependent dephosphorylation of the receptor (2).

DISCUSSION

5-HT$_{2C}$ Rs are implicated in physiological processes such as cerebrospinal fluid production as well as illnesses and disorders including anxiety, migraines, and eating and sleeping disorders (27, 28). Furthermore, the 5-HT$_{2C}$ R is a target for hallucinogenic drugs such as lysergic acid diethylamide (29, 30). A thorough understanding of intracellular signaling by the 5-HT$_{2C}$ R, including its interaction with PDZ domain containing proteins, may give insight into the cellular mechanisms that underlie diverse physiological processes. PDZ domain-containing proteins are involved in the localization of potassium channels and glutamate receptors in the synapse as well as localization of numerous other receptors and proteins (31–36). PDZ domains typically bind to the carboxyl termini of target proteins that contain a PDZ binding motif. Interestingly, some PDZ binding motifs have been shown to be phosphorylated (37–47), including the 5-HT$_{2C}$ R (2).
Phosphorylation Regulates PDZ-5-HT\textsubscript{2C} Receptor Interaction

In the current paper, 5-HT\textsubscript{2C} R interaction with PDZ 10 of MUPP1 was independently confirmed by using pull-down and protein overlay assays. These results are consistent with the report by Becamel et al. (18) that demonstrated PDZ 10 interaction with the 5-HT\textsubscript{2C} R in yeast two-hybrid assays. Becamel et al. (18) also demonstrated that mutation of the critical residues of the PDZ motif on the receptor, Ser\textsuperscript{458} or Val\textsuperscript{460} to alanine, abolished interaction with PDZ 10. Similarly, our results also document that the carboxyl-terminal residues of the 5-HT\textsubscript{2C} R are necessary for MUPP1 interaction; truncated 5-HT\textsubscript{2C} R tail proteins missing the terminal PDZ binding motif failed to show detectable interaction with MUPP1. There are four recognized classes of carboxyl-terminal PDZ binding motifs to date (48). The carboxyl terminus of the 5-HT\textsubscript{2C} R is predicted to be a critical residue for interacting with the PDZ 10 domain backbone. Since both Ser\textsuperscript{458} and Ser\textsuperscript{459} of the 5-HT\textsubscript{2C} R are phosphorylated upon ligand activation (2), we examined the role of receptor phosphorylation on the 5-HT\textsubscript{2C} R-MUPP1 interaction.

In the next series of experiments, we investigated whether mutations of the terminal serines to aspartates that mimic phosphorylation of serines would affect binding of the 5-HT\textsubscript{2C} R to PDZ 10 of MUPP1. S458D and S458D/S459D mutations led to a significant loss of receptor tail interaction to PDZ 10. These data are in agreement with crystal structure data indicating that the hydroxyl group of the Ser at the −2 position of a type I PDZ binding target is important for hydrogen bonding to His at the first position of the α B helix (α B1) of a type I PDZ domain such as PDZ 10 (21). Our data suggest that the introduction of a negatively charged group at the −2 position of the receptor tail disrupts the hydrogen bonding to the PDZ 10 domain resulting in a loss of interaction. The S459D mutation, on the other hand, retains wild-type interaction. This result is also consistent with structural studies showing that the side chain of the −1 position residue of a PDZ binding target does not hydrogen-bond to residues in the PDZ domain backbone but rather points away from the pocket (21). Interestingly, c-Kit, a receptor tyrosine kinase whose carboxyl-terminal sequence ends in Asp-Asp-Val, and claudin-1, a tight junction protein whose carboxyl-terminal sequence ends in Asp-Tyr-Val, have been identified to interact with PDZ 10 of MUPP1 (49, 50). These observations are unexpected in light of structural information on PDZ-ligand complexes, including the current data of carboxyl-terminal mutants in the 5-HT\textsubscript{2C} R receptor. One possible explanation is that c-Kit and claudin-1 interaction with PDZ 10 may involve additional residues upstream from the carboxyl termini.

To determine whether serotonin stimulation within a cellular context would alter 5-HT\textsubscript{2C} R binding to PDZ 10, we examined the receptor stably expressed in NIH-3T3 cells in the absence or presence of serotonin. A dose-dependent loss of MUPP1 interaction for this receptor was previously observed by Westphal et al. (51) when cells were metabolically labeled with \textsuperscript{32}P orthophosphoric acid. Alternatively, an unidentified protein that attenuates MUPP1 binding to the receptor may be reduced by phosphatase treatment and account for this observation. When receptor was stimulated with a maximal serotonin antagonist, BOL. Moreover, alkaline phosphatase treatment reversed the effect of serotonin. Interestingly, in the absence of serotonin, phosphatase treatment also resulted in increased receptor binding to PDZ 10. We speculate that this may be indicative of constitutive phosphorylation; basal phosphorylation for this receptor was previously observed by Westphal et al. (51) when cells were metabolically labeled with \textsuperscript{32}P orthophosphoric acid. Alternatively, an unidentified protein that attenuates MUPP1 binding to the receptor may be reduced by phosphatase treatment and account for this observation. When receptor was stimulated with a maximal serotonin-mediated receptor phosphorylation dose, agonist washout experiments demonstrated that receptor interaction with MUPP1 was restored in a time-dependent manner. We speculate that this restored binding between the receptor and MUPP1 is the result of endogenous phosphatases. Results from antagonist, phosphatase treatments, and agonist washout experiments demonstrated that the kinase responsible for phosphorylation of the 5-HT\textsubscript{2C} R has been initiated. Initial studies suggest that the usual second messenger-activated kinases are not involved (data not shown). This finding is consistent with investigations of another G-
Regulates the coupling of the 5-HT2C receptor to various effectors to assemble nature of PDZ proteins, we propose that MUPP1 sensitive to basal phosphorylation of the receptor. Due to the PDZ 10, resulted in a decrease in MUPP1 interaction. We also shown to phosphorylate the β₂- and β₂-agonist receptors at carboxyl-terminal serines of a PDZ binding motif, which reduces their interaction with PDZ domain proteins (39, 40, 54). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)glutamate receptor exhibits dual specificity for its interacting partners PICK1 (protein interacting with C kinase) and GRIP/ABP (glutamate receptor-interacting protein)/ (AMPA receptor-binding protein), depending upon whether the glutamate receptor 2/3 subunit is phosphorylated by protein kinase C (42–45, 55, 56). Finally, the binding of stargazin, a transmembrane protein that associates with AMPA receptors, to PDZ-95 is regulated by protein kinase A phosphorylation (46, 47, 57, 58).

In this paper, we established that phosphorylation of the 5-HT₂C R at Ser⁴⁵⁸, the serine residue critical for binding to PDZ 10, resulted in a decrease in MUPP1 interaction. We also provide evidence that receptor interaction with MUPP1 is sensitive to basal phosphorylation of the receptor. Due to the assembling nature of PDZ proteins, we propose that MUPP1 regulates the coupling of the 5-HT₂C R to various effectors to activate downstream signaling processes. MUPP1 interaction with the 5-HT₂C R when the receptor is in an unphosphorylated form may keep the receptor in a conformation state (and vice versa) that is masked from some of its downstream signaling partners. Then in an agonist-dependent or -independent manner (an event that is not fully understood), the receptor becomes phosphorylated at the PDZ binding motif, and MUPP1 is released from the receptor, resulting in a change in conformation that reveals downstream signaling molecules scaffolded to MUPP1. Changes in MUPP1 folding have been postulated as a result of observations by Becamal et al. (18), showing that when MUPP1 is expressed alone in COS cells, a carboxyl-terminal vesicular stomatitis virus epitope tag is not accessible; however, when MUPP1 is co-expressed with the 5-HT₂C R, the tag is accessible. The possibility also exists that the sole function of MUPP1 may be to simply traffic or anchor the 5-HT₂C R to specific membrane domains without a direct effect upon receptor signaling. Based upon the data presented here and previous studies on PDZ proteins and receptors, phosphorylation appears to be a critical regulator of PDZ protein-protein interactions, including regulation of the interaction between MUPP1 and the 5-HT₂C R. Our results suggest that cells may have an underlying mechanism to dynamically regulate overall cellular activity in the absence and subsequent exposure to agonist by modulating basal receptor phosphorylation and thereby balancing the extent of maximal receptor activation.

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