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Interaction of Serotonin 5-Hydroxytryptamine Type 2C Receptors with PDZ10 of the Multi-PDZ Domain Protein MUPP1*

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By using the yeast two-hybrid system, we previously isolated a cDNA clone encoding a novel member of the multivalent PDZ protein family called MUPP1 containing 13 PDZ domains. Here we report that the C terminus of the 5-hydroxytryptamine type 2C (5-HT_{2C}) receptor selectively interacts with the 10th PDZ domain of MUPP1. Mutations in the extreme C-terminal SSV sequence of the 5-HT_{2C} receptor confirmed that the S*X*V motif is critical for the interaction. Co-immunoprecipitations of MUPP1 and 5-HT_{2C} receptors from transfected COS-7 cells and from rat choroid plexus verified this interaction *in vivo*. Immunocytochemistry revealed an S*X*V motif-dependent co-clustering of both proteins in transfected COS-7 cells as well as a colocalization in rat choroid plexus. A 5-HT_{2C} receptor-dependent unmasking of a C-terminal vesicular stomatitis virus epitope of MUPP1 suggests that the interaction triggers a conformational change within the MUPP1 protein. Moreover, 5-HT_{2A} and 5-HT_{2B}, sharing the C-terminal E*X*(V/I)S*X*V sequence with 5-HT_{2C} receptors, also bind MUPP1 PDZ domains *in vitro*. The highest *MUPP1* mRNA levels were found in all cerebral cortical layers, the hippocampus, the granular layer of the dentate gyrus, as well as the choroid plexus, where 5-HT_{2C} receptors are highly enriched. We propose that MUPP1 may serve as a multivalent scaffold protein that selectively assembles and targets signaling complexes.

There is ample evidence suggesting that the function of a receptor is dependent on its specific subcellular localization. Sequence-specific interactions between proteins provide the basis for the structural and functional organization of receptors within cells. For a few members of the G-protein-coupled receptor family, these interactions have been described to be mediated by C-terminal interactions with PDZ (PSD-95/discs large/ZO-1) domain-containing proteins. The best investigated example is the β_2-adrenergic receptor, which interacts with the Na^+/H^+ exchanger regulatory factor (NHERF/EBP50)[1] (1). The interaction of NHERF with the β_2-adrenergic receptor is mediated via binding of the first PDZ domain of NHERF to the extreme C terminus of the β_2-adrenergic receptor in an agonist-dependent manner, thereby regulating Na^+/H^+ exchange (2). NHERF has also been described to link proteins with the actin cytoskeleton through association with ERM (ezrin-radixin-moesin) proteins (3). In fact, the PDZ-mediated interaction of the β_2-adrenergic receptor with NHERF family proteins has been shown to control recycling of internalized β_2-adrenergic receptors. Disrupting the β_2-adrenergic receptor-NHERF interaction perturbs the endocytic sorting of the β_2-adrenergic receptor, resulting in lysosomal degradation (4).

C-terminal interactions of G-protein-coupled receptors with PDZ domain-containing proteins extends to a member of the somatostatin receptor family (sst). The subtype sst2 interacts selectively with a highly homologous PDZ domain contained within the protein CortBP1/ProSAP1/Shank2 (5) and the recently cloned synaptic protein SSTRIP (somatostatin receptor-interacting protein)/Spank1/synamon/Shank1 (6). Both proteins belong to a common family recently termed Shanks (7, 8) or ProSAP (9), sharing essentially identical domain structures such as ankyrin repeats, an SH3 domain, the PDZ domain, a sterile α motif domain, and a proline-rich region that links Shanks to cortactin, a constituent of the actin cytoskeleton (10).

5-HT_{2C} receptors are broadly expressed in the central nervous system and in the choroid plexus (11, 12) and are involved in a diversity of physiological functions such as the control of nociception, motor behavior, endocrine secretion, thermoregulation, modulation of appetite, and the control of exchanges between the central nervous system and the cerebrospinal fluid (13–17). These receptors contain a C-terminal sequence, SSV* (where the asterisk indicates a carboxyl group), corresponding to the T/S*X*V* motif. This motif is potentially implicated in protein-protein interactions with PDZ domains as originally described for the C termini of the *N*-methyl-D-aspartate receptor and K^+ channel subunits (18, 19). The T/S*X*V* motif is also present in C termini of various other G-protein-coupled receptors, including the 5-HT_{2A} (18) and human 5-HT_{2B} receptors. By virtue of its interaction with the C terminus of the 5-HT_{2C} receptor, we recently isolated a novel cDNA encoding MUPP1, a protein with 13 PDZ domains (20). MUPP1 belongs to the family of multi-PDZ proteins comprising CIPP (channel-interacting PDZ domain protein) (21, 22), INADL (INAD-like protein) (23), and a putative *Caenorhabditis elegans* polypeptide referred to as C52A11.4 (20), containing 4, 7, or 10 PDZ do-

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[1] The abbreviations used are: NHERF, Na^+/H^+ exchanger regulatory factor; 5-HT_{2C}, 5-hydroxytryptamine type 2C; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; VSV, vesicular stomatitis virus; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid.

mains, respectively, and no other obvious catalytic domain. PDZ domains highly similar to those of MUPP1 are arrayed in the same order in all four proteins, implying the requirement of a precise arrangement for the assembly into a functional macromolecular complex (20). The present work provides biochemical and immunohistochemical evidence that the 5-HT$_{2C}$ receptor interaction with MUPP1 takes place *in vitro* and *in vivo*. Among the 13 PDZ domains of MUPP1, 5-HT$_{2C}$ receptors exclusively interact with the 10th PDZ domain, emphasizing the high selectivity of PDZ domain interactions. Moreover, the interaction induces a conformational change in the MUPP1 molecule and a co-clustering that might trigger a downstream signal transduction pathway.

EXPERIMENTAL PROCEDURES

Two-hybrid Screening—Yeast two-hybrid screening was performed using the CG1945 strain (24) harboring the *HIS3* and β-galactosidase reporter genes under the control of upstream *GAL4*-binding sites (CLONTECH). The yeast culture was transformed using the polyethylene glycol/LiAc method (25). Interactions between bait and prey were monitored by β-galactosidase activity in colonies transferred onto Hybond N filters (Amersham Pharmacia Biotech, Freiburg, Germany).

Domain Analysis of the Interaction—To assemble the full-length clone pBSKSII-rMUPP1, a PCR fragment covering region −183 to 2000 with a silent mutation of C^{1979} to T to delete a *Bam*HI site was generated by reverse transcription-PCR from rat femoral muscle cDNA that was prepared as described (26) using recombinant *Pfu* polymerase (Stratagene, La Jolla, CA). The resulting 5′-end was ligated into the remaining *Bam*HI site of the partial rat *MUPP1* cDNA (pXMD1/rMUPP1) (20). pBSKSII-rMUPP1 was sonicated to an average size of 500 base pairs, and blunt-ended DNA fragments were cloned into the *Srf*I site of pCR-Script (Stratagene). The inserts of 20,000 recombinant plasmids retrieved by *Not*I and *Eco*RI digestion were directionally cloned into the *Xho*I and *Eco*RI sites of pACT-2 using *Not*I/*Xho*I adapter oligonucleotides. The resulting library was transformed into yeast strain CG1945 carrying pAS2-1/hu2C. pAS2-1/hu2C was described previously (20). The resulting plasmid fuses amino acids 369–458 of human 5-HT$_{2C}$ receptors to the Gal4 DNA-binding domain (amino acids 1–147) of yeast Gal4 in the yeast expression vector pAS2-1 (CLONTECH). Transformants selected for *HIS3* expression were picked after 5 days. Plasmid DNA was extracted, and inserts were retrieved by PCR with plasmid-specific primers, sequenced, and aligned with the rat *MUPP1* cDNA sequence.

Mutagenesis—The 90-amino acid C-terminal fragment of the human 5-HT$_{2C}$ receptor was mutagenized by PCR using reverse primers harboring nucleotide exchanges and by using pAS2-1/hu2C as template. The amplified DNA was directionally subcloned into the *Bam*HI and *Eco*RI sites of pBluescript KSII (Stratagene) and sequenced. The inserts were transferred into either pAS2-1 (*Bam*HI and *Sal*I) or pGEX-3X (*Bam*HI and *Eco*RI; Amersham Pharmacia Biotech). The mutant plasmid pRK5/h5-HT$_{2C\text{-}SSA}$ was obtained by PCR using a reverse primer encoding the V458A mutation by a GCG codon using pRK5/h5-HT$_{2C}$ as template.

In Vitro Protein-Protein Interaction—The partial cDNA of human MUPP1 was amplified by PCR from pACT-2/huMUPP1 (clone 1) (20) and inserted directionally into the *Hin*dIII and *Eag*I sites of pBAT (27). *In vitro* translation of [^{35}S]methionine-labeled MUPP1 was performed using T3 RNA polymerase with the TNT coupled reticulolysate lysate system (Promega, Mannheim, Germany) according to the manufacturer's instructions. The C-terminal tails of the 5-HT$_{2A}$ (residues 380–471), 5-HT$_{2B}$ (residues 414–481), and 5-HT$_{2C}$ (residues 368–458) receptors were subcloned into pGEX-3X, giving rise to plasmids pGEX2A92, pGEX2B67 and pGEX2C90, respectively. Synthesis of recombinant proteins (GST-2A92 and GST-2C90) in BL21 cells (Amersham Pharmacia Biotech) was induced by 0.25 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 30 °C. GST-2B67 was induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 25 °C. Cells were sonicated in buffer S (20 mM HEPES (pH 7.9), 100 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and pelleted in buffer S, 1% Triton, and 10% glycerol. GST proteins were purified on bulk glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions, with the exception of using buffer S instead of PBS. GST proteins were eluted with buffer S, 10% glycerol, and 10 mM glutathione and dialyzed against buffer S and 10% glycerol (2 × 3 h at 4 °C). ^{35}S-Labeled MUPP1 was incubated in buffer S, 10% glycerol, and 1% Nonidet P-40 with glutathione-Sepharose 4B beads saturated with 10 μg of GST fusion protein or GST for 3.5 h at 4 °C in the presence or absence of 1.5 mM synthetic peptide VVSERISSV or control peptide VVSERIASA (B&G Biotech GmbH, Freiburg). Beads were washed four times for 10 min in buffer S, 10% glycerol, and 1% Nonidet P-40. Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

Expression Plasmids—pBSKSII-rMUPP1 was cut with *Eco*RI and *Xho*I and ligated with a PCR product containing a C-terminal VSV tag flanked by two *Xho*I sites into pXMD1 (28), generating pXMD1/rMUPP-VSV. pBSKSII-rMUPP1 was cut with *Nde*I and *Sal*I and ligated into pCI-neo (Promega) *Eco*RI and *Sal*I sites using adapter oligonucleotides containing the VSV tag (YTDIEMNRLGK) DNA sequence (TACACCGATATCGAGATGAACAGGCTGGGAAAGTGA). The resulting plasmid, pCI-neo/VSV-ΔMUPP1, fuses the N-terminal VSV tag and amino acids 1337–2055 of the rat MUPP1 protein. pRK5/h5-HT$_{2C}$ was directionally subcloned from pXMD1/h5-HT$_{2C}$ (29) by releasing the cDNA insert with the restriction digest enzymes *Eco*RI and *Xba*I. Using PCR, the human 5-HT$_{2C}$ receptor cDNA was N-terminally tagged with a stretch of nucleotides (ATGGAACAAAAGCTTATTTCTGAAGAAGACTTG) encoding a 10-amino acid epitope (EQKLISEEDL) of the human c-Myc protein (30). The amplified DNA was directionally subcloned into the *Eco*RI and *Xba*I sites of pRK5, yielding pRK5/c-Myc-hu5-HT$_{2C}$.

Cell Culture and Transfection—Expression plasmids were introduced into COS-7 cells by electroporation as described (31). Briefly, cells were trypsinized, centrifuged, and resuspended in electroporation buffer (50 mM K_2HPO_4, 20 mM CH_3CO_2K, 20 mM KOH, and 26.7 mM $MgSO_4$ (pH 7.4)) with 1 μg of pRK5/h5-HT$_{2C}$ and 1.5 μg of pXMD1/rMUPP-VSV. The total amount of DNA was kept constant at 15 μg by filling up with pRK5 DNA. After 15 min at room temperature, 10^7 cells were transferred to a 0.4-cm electroporation cuvette (Bio-Rad) and pulsed using a Gene Pulser apparatus (setting at 1000 microfarads and 280 V). Cells were resuspended in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% dialyzed fetal bovine serum (Life Technologies, Inc.) and plated on 10-cm Falcon Petri dishes or into 12-well clusters.

Antibodies—The production, characterization, and purification of the rabbit polyclonal 522 antibody raised against the mouse 5-HT$_{2C}$ receptor have been described (32). The mouse monoclonal anti-c-Myc antibody was a gift from B. Mouillac. Rabbit polyclonal anti-MUPP1 antiserum was raised against GST fusion protein containing the third PDZ domain of rat MUPP1 (residues 318–451) (antibody 2324) or amino acids 780–1063 of human MUPP1, containing the region between the fourth and fifth PDZ domains (antibody 2526).[2] The mouse monoclonal anti-VSV antibody was purchased from Sigma. The secondary antibodies used were Oregon green-conjugated goat anti-rabbit or anti-mouse antibody and Texas Red-conjugated goat anti-mouse or anti-rabbit IgG antibody (all from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Membrane Preparations and Immunoprecipitation—Rat choroid plexus or COS-7 cells were briefly centrifuged (3 min at 200 × *g*), and pellets were resuspended in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, and protease inhibitor mixture (2.5 μg/ml each leupeptin, aprotinin, and antipain and 0.5 mM benzamidine (pH 7.4))), homogenized 20 times with a glass-Teflon homogenizer at 4 °C, and centrifuged at 100,000 × *g* for 1 h. Each membrane pellet was resuspended in CHAPS extraction buffer (50 mM Tris-HCl (pH 7.4) containing 0.05 mM EDTA, 10 mM CHAPS, and protease inhibitor mixture (see above)) for 2 h in rotation at 4 °C. After centrifugation (1 h at 100,000 × *g*), CHAPS-soluble proteins were incubated overnight at 4 °C with 2 μl of anti-5-HT$_{2C}$ receptor 522 antibody, anti-VSV antibody, or anti-MUPP1 antiserum for the immunoprecipitation. 50 μl of protein A-Sepharose beads (Sigma) was added to the supernatant, and the mixture was then rotated at 4 °C for 1 h. After five washes in CHAPS-free buffer, the immunoprecipitates and co-immunoprecipitates were dissociated in Laemmli sample buffer. The samples were centrifuged, and the supernatants were fractionated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel, electrotransferred to nitrocellulose membrane (Hybond C extra, Amersham Pharmacia Biotech), probed with rabbit anti-5-HT$_{2C}$ receptor antibody or anti-MUPP1 antiserum (1:500), and then detected by enhanced the chemiluminescence method (Renaissance Plus, PerkinElmer Life Sciences).

Determination of Inositol Phosphate Accumulation—6 h after transfection, cells were incubated overnight in serum-free Dulbecco's modified Eagle's medium with 1 μCi/ml *myo*-[^{3}H]inositol (17 Ci/mol; PerkinElmer Life Sciences). Total inositol phosphate accumulation in

[2] S. Poliak and E. Peles, manuscript in preparation.

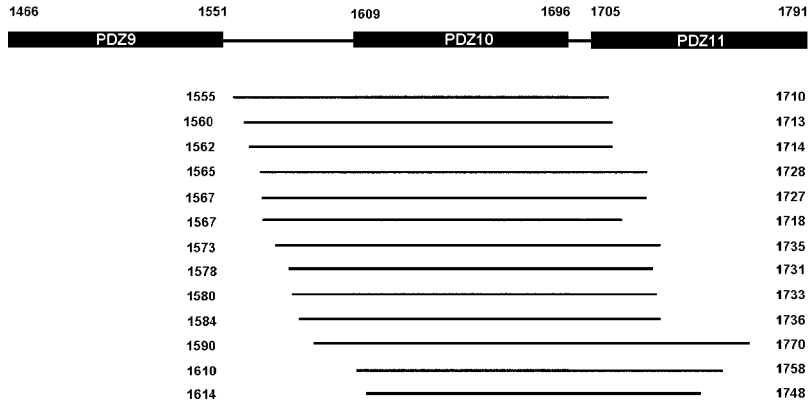


FIG. 1. **The C-terminal domain of the 5-HT$_{2C}$ receptor interacts with PDZ10 of MUPP1.** Randomly generated MUPP1 fragments interacting in the yeast two-hybrid system with the C-terminal sequence of the 5-HT$_{2C}$ receptor are displayed relative to the domain map and are identified by amino acid numbers. All selected clones share the PDZ10 coding sequence (*shaded*).

response to serotonin in the presence of 10 mM LiCl for 10 min at 37 °C was determined as described (33). The amount of [^{3}H]inositol phosphate formed was separated according to the ion exchange method (34). Concentration-response curves, EC_{50} values, and Scatchard analysis were calculated with Synergy software.

Membrane Preparations and Radioligand Binding Assay—Membranes were prepared from transiently transfected COS-7 cells. 6 h after transfection, cells were incubated overnight in serum-free Dulbecco's modified Eagle's medium. Cells were washed twice in PBS, scraped with a rubber policeman, harvested in PBS, and centrifuged at 4 °C for 4 min at 200 × *g*. The pellet was resuspended in 10 mM HEPES (pH 7.4), 5 mM EGTA, 1 mM EDTA, and 0.32 M sucrose and homogenized 10 times with a glass-Teflon homogenizer at 4 °C. The homogenate was centrifuged at 100,000 × *g* for 20 min, and the membrane pellet was resuspended in 50 mM HEPES (pH 7.4) and stored at −80 °C until used. 5-HT$_{2C}$ receptor densities were estimated using the specific radioligand [*N*^{6}-*methyl*-^{3}H]mesulergine at a saturating concentration of 4 nM. Mianserin (1 μM) was used to determine nonspecific binding. Protein concentrations were determined using the Bradford protein assay (Bio-Rad).

Immunocytochemistry—Cells were grown on 35-mm dishes, fixed 24 h after transfection in 4% paraformaldehyde and PBS (pH 7.4) for 20 min at room temperature, washed three times in 0.1 M glycine buffer (pH 7.4), and permeabilized with 0.05% Triton X-100 for 5 min. Cells were washed in 0.2% gelatin and PBS and incubated overnight at 4 °C with the primary antibody diluted 1:500 in 0.2% gelatin and PBS. Cells were washed and incubated for 1 h at room temperature with the secondary antibody diluted 1:1000 in 0.2% gelatin and PBS. Cultures were washed, mounted on glass slides using Gel Mount (Biomeda Corp., Foster City, CA), and viewed on a Zeiss Axioplan 2 microscope (Zeiss, Göttingen, Germany).

Enzyme-linked Immunosorbent Assay—Cells were seeded at a density of 8 × 10^5 cells/well on a 12-well plate, fixed 24 h after transfection in 4% paraformaldehyde and PBS (pH 7.4) for 20 min at 4 °C, and washed three times in 0.1 M glycine buffer (pH 7.4). Cells were incubated for 5 min in 3% H_2O_2 and PBS to minimize endogenous peroxidase activity. The anti-c-Myc antibody (diluted 1:500 in PBS and 0.2% bovine serum albumin) was applied for 1.5 h at 37 °C. Plates were rinsed five times with PBS and incubated for 1 h at 37 °C with horseradish peroxidase-conjugated anti-mouse antibody diluted 1:250 in PBS and 0.2% bovine serum albumin. Plates were rinsed seven times with PBS, and the reaction was developed by adding the substrate ABTS. Absorbance was measured at 410 nm using an enzyme-linked immunosorbent assay reader. Control plates without cells were included to determine background activity, which was subtracted from the A_{410} readings. Each experiment was performed in quadruplicates.

Immunohistochemistry—Rats were deeply anesthetized with pentobarbital and transcardially perfused with a fixative solution containing 4% paraformaldehyde and 0.1 M PBS (pH 7.4). Brains were removed, post-fixed at 4 °C for 2 h in the same fixative solution, and stored overnight at 4 °C in PBS containing 30% sucrose for cryoprotection. Sections of 10 μm were cut with a cryostat (Microm HM500), collected on slides, and then stored at −80 °C. Prior to the experiment, sections were rinsed serially for 5 min once with PBS containing 20% sucrose, 10% sucrose, and without sucrose, respectively. Preincubation was performed in 0.25% Triton X-100, 20% horse serum, and PBS (pH 7.4) for 1 h at room temperature. Sections were incubated with either the rabbit polyclonal anti-5-HT$_{2C}$ receptor 522 antibody or affinity-purified anti-MUPP1 2324 or 2526 antibody diluted 1:500 in 1% horse serum and PBS overnight at 4 °C. After three washes in PBS, sections were incubated with donkey Cy3-conjugated anti-rabbit IgG antibody (1:2000; Jackson ImmunoResearch Laboratories, Inc.) in 1% horse serum and PBS at 4 °C for 3 h. Sections were rinsed with PBS and mounted on slides, which were coverslipped with Mowiol (Calbiochem).

In Situ Hybridization—Rats (10-week-old Wistar male adult) were killed by decapitation, and their brains were removed and frozen immediately on dry ice. Microtome cryostat sections (20 μm) were thaw-mounted onto gelatin-coated slides, air-dried, and kept at −20 °C until used. Hybridization was performed at 60 °C essentially as described (35) using radioactive [α-^{33}P]UTP-labeled riboprobes that were prepared as follows. The plasmid pXMD1/rMUPP1 (20) was cut with *Sma*I to release a fragment of 2669 base pairs covering the region from 4653 to the 3′-end of the rat *MUPP1* cDNA, which was ligated into the *Srf*I site of pCR-Script. For *in vitro* transcription, both sense and antisense constructs were linearized with *Xho*I and transcribed using T3 RNA polymerase. RNA synthesis was performed in a 20-μl reaction with 1 μg of linearized plasmid DNA, 10 mM dithiothreitol, 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.1 mM UTP, 70 μCi of [α-^{33}P]UTP, 40 units of RNasin (Promega), and 20 units of RNA polymerase (Roche Molecular Biochemicals, Mannheim) and incubated for 1 h at 37 °C. Synthesis was continued with the addition of 20 units of RNasin and 20 units of RNA polymerase for 1 h. DNA was digested with 10 units of RQ1 RNase-free DNase (Promega) for 20 min. The reaction was stopped with 5 μl of EDTA (0.5 M) and applied to a Sephadex G-50 spin column (Roche Molecular Biochemicals). RNA was hydrolyzed in 20 mM $NaHCO_3$ and 30 mM $NaCO_3$ for 20 min and neutralized in 3.3 mM HCl.

RESULTS

Characterization of the PDZ Domain Interaction—It is evident that PDZ domains display sufficient variability to allow distinct protein-protein interactions (36, 37). Since rat MUPP1 has 13 PDZ domains, we analyzed the interaction of all parts of MUPP1 with the C terminus of the 5-HT$_{2C}$ receptor. A library of fusion proteins with the Gal4 activation domain was constructed through a random generation of ~500-base pair DNA fragments by sonication of pBSKSII-rMUPP1. This tagged fragment library was cotransfected into yeast with the vector encoding the C terminus of the 5-HT$_{2C}$ receptor attached to the Gal4 DNA-binding domain, and resultant yeast colonies were selected by histidine starvation. DNAs from 13 selected colonies were amplified by PCR with plasmid-specific primers, and the amplified DNAs were sequenced. All sequences shared the entire MUPP1 PDZ10 coding region (Fig. 1), indicating a selective interaction of the 5-HT$_{2C}$ receptor C terminus with PDZ10. The complete PDZ10 domain seems to be required since PDZ10 was complete in all selected clones.

Characterization of the 5-HT$_{2C}$ Receptor C-terminal Interaction—Ion channels such as the *N*-methyl-D-aspartate and Shaker-type K^+ channels share a C-terminal T/S*X*V* motif known to interact with PDZ domains (18, 19). The 5-HT$_{2C}$ receptor has a similar C-terminal SSV* sequence that may define a part of the critical motif for interaction. To determine whether the 3 C-terminal amino acid residues in the 5-HT$_{2C}$ receptor are essential for binding to the PDZ10 domain of MUPP1, mutational analysis was performed in which the res-

TABLE I
Sequence requirements in the 5-HT$_{2C}$ receptor carboxyl terminus mediating interaction with MUPP1

Single amino acid substitutions are shown in boldface. The ability to interact with MUPP1 was tested in the yeast two-hybrid assay. *HIS3* activity was measured by the percentage of colonies growing on histidine-lacking medium: ++, >60%; +, 30–60%; −, no significant growth. β-Galactosidase (β-Gal) activity was determined from the time taken for colonies to turn blue in the 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) filter lift assay: ++, <3 h; +, 3–6 h; −, no significant β-galactosidase activity.

5-HT$_{2C}$ carboxyl terminus (369–458)	*HIS3*	β-Gal
369-SSV (wild-type)	++	++
369-**T**SV	+	++
369-**A**SV	−	−
369-S**A**V	+	++
369-SS**A**	−	−

idue at each position was replaced with an alanine, and interactions with MUPP1 were tested in the yeast two-hybrid assay. A partial human MUPP1 clone (clone 1) (see Ref. 20) encoding the C-terminal 454 amino acids including PDZ10–13 served as bait. Substitution of Ser^{456} and Val^{458} with Ala abolished the interaction with MUPP1 (Table I). In contrast, the conservative mutation of Ser^{456} to Thr and the mutation of Ser^{457} to Ala were tolerated, although the resultant yeast clones displayed reduced growth on histidine-lacking medium. To independently demonstrate the interaction of MUPP1 with the 5-HT$_{2C}$ receptor C terminus, fusion proteins of either the wild-type form or various mutants of C-terminal 5-HT$_{2C}$ receptor sequences (90 amino acids) fused to GST were bound to glutathione-Sepharose (Fig. 2*A*) and incubated with *in vitro* translated ^{35}S-labeled MUPP1 (clone 1, PDZ10–13). MUPP1 bound to the GST-5-HT$_{2C}$ fusion proteins was resolved on an SDS-polyacrylamide gel and visualized by autoradiography. Only the S457A mutant (Fig. 2*A*, *fifth lane*) retained MUPP1-binding activity, as observed in the yeast two-hybrid assay (Table I), which confirmed the S*X*V motif as a critical determinant for the PDZ domain interaction.

Interaction of MUPP1 with 5-HT$_{2A}$ and 5-HT$_{2B}$ Receptors—Multiple sequence alignment of the C-terminal ends from all members of the 5-HT$_2$ receptor family (Fig. 2*B*) revealed a common C-terminal amino acid sequence motif, E*X*(V/I)S*X*V*. We therefore analyzed whether the 5-HT$_{2A}$ or 5-HT$_{2B}$ receptor C terminus would also interact with MUPP1. GST fusion proteins of the C-terminal 92 amino acids of the 5-HT$_{2A}$ receptor (GST-2A92) and the C-terminal 67 amino acids of the 5-HT$_{2B}$ receptor (GST-2B67) were bound to glutathione-coupled Sepharose beads and incubated with *in vitro* translated ^{35}S-labeled MUPP1 (clone 1, PDZ10–13). MUPP1 bound to both the GST-2A92 (Fig. 2*C*, *second lane*) and GST-2B67 (*fourth lane*) fusion proteins, but not to GST alone (*first lane*). Specificity of the interaction with MUPP1 PDZ domains was demonstrated by co-incubation with a 9-amino acid synthetic peptide mimicking the 5-HT$_{2C}$ receptor C terminus, which prevented MUPP1 binding to all three GST-5-HT$_2$ receptor fusion proteins (Fig. 2*C*, *third*, *fifth*, and *seventh lanes*). A control peptide harboring the S456A and V458A mutations did not compete with MUPP1 binding (data not shown), confirming the requirement of Ser (position −2) and Val (position 0) for PDZ domain interaction.

Interaction of Heterologously Expressed 5-HT$_{2C}$ Receptors with MUPP1—To determine whether MUPP1 forms a protein complex with the 5-HT$_{2C}$ receptor in living cells, COS-7 cells were transfected with cDNA encoding the human 5-HT$_{2C}$ receptor in the presence or absence of the C-terminally VSV-tagged rat MUPP1 protein (MUPP1-VSV). In a CHAPS-soluble cell extract, the anti-5-HT$_{2C}$ receptor 522 antibody (11) revealed two bands between 60 and 50 kDa, corresponding to the glycosylated and unglycosylated 5-HT$_{2C}$ receptor, respectively (Fig. 3 *first* and *fifth lanes*). In fact, treatment with *N*-glycosidase F to remove *N*-linked sugars caused a shift of the upper band to the level of the lower band (data not shown). CHAPS-soluble extracts were immunoprecipitated by an anti-VSV antibody and then immunoblotted with the anti-5-HT$_{2C}$ receptor 522 antibody. 5-HT$_{2C}$ receptors were co-immunoprecipitated by MUPP1-VSV from cells cotransfected with 5-HT$_{2C}$ receptor and MUPP1 expression plasmids (Fig. 3, *second lane*). This indicates that the MUPP1 protein and the 5-HT$_{2C}$ receptor are able to interact when expressed in a heterologous system. In contrast, when cells were transfected with the 5-HT$_{2C}$ receptor alone, the receptor was not revealed in the immunoprecipitates (Fig. 3, *sixth lane*), indicating the specificity of the co-immunoprecipitation. The mutant 5-HT$_{2C}$ receptor construct in which the C-terminal Val of the S*X*V PDZ-binding motif was replaced with Ala (V458A, 5-HT$_{2C\text{-}SSA}$) was extracted in amounts similar to those of the native 5-HT$_{2C}$ receptor, but to a higher extent in the glycosylated form (Fig. 3, *third lane*). When cells expressed MUPP1-VSV and the 5-HT$_{2C\text{-}SSA}$ receptor, the mutant receptor could not be immunoprecipitated with the anti-VSV antibody (Fig. 3, *fourth lane*). This is in accordance with the *in*

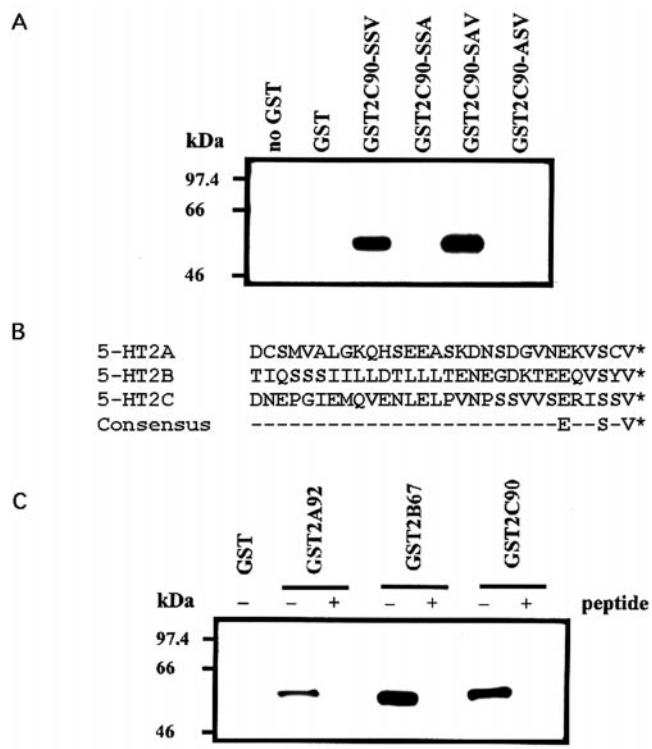


FIG. 2. ***In vitro* binding of C-terminal receptor sequences to MUPP1.** DNA encoding C-terminal receptor sequences in-frame with the GST moiety were bacterially expressed and purified on glutathione-Sepharose. GST pull-down reactions were performed with *in vitro* translated ^{35}S-labeled MUPP1 (clone 1, PDZ10–13), and the adsorbed proteins were separated by SDS-polyacrylamide gel electrophoresis. MUPP1 that was copurified with the fusion protein was identified by autoradiography. *A*, analysis of GST fusion proteins that contained 90-amino acid C-terminal 5-HT$_{2C}$ receptor sequences from either the wild-type form (GST-2C90-SSV) or mutants in which 1 of the last 3 amino acids was replaced by alanine as indicated. *B*, alignment of the C-terminal 30 amino acids of human 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors. All sequences share the C-terminal E*X*(V/I)S*X*V* motif, where the asterisk indicates a carboxyl group. *C*, analysis of the GST fusion protein with the C-terminal 92 amino acids of the 5-HT$_{2A}$ receptor sequence (GST-2A92) or the C-terminal 67 amino acids of the 5-HT$_{2B}$ receptor (GST-2B67) or GST-2C90. Where indicated, an 8.5-fold excess of the E*X*(V/I)S*X*V sequence-containing peptide was present during incubation.

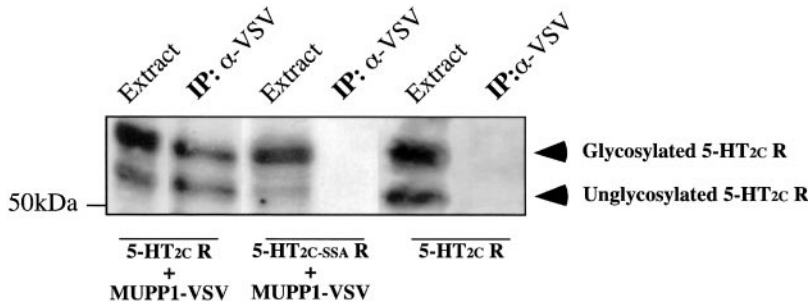


FIG. 3. **Co-immunoprecipitation of MUPP1 and 5-HT$_{2C}$ receptors from transfected COS-7 cells.** Cells were transfected with MUPP1-VSV and the 5-HT$_{2C}$ receptor (*5-HT2C R*) (*first* and *second lanes*), MUPP1-VSV and the mutant 5-HT$_{2C\text{-}SSA}$ receptor (*third* and *fourth lanes*), or the 5-HT$_{2C}$ receptor alone (*fifth and sixth lanes*). Membrane fractions were solubilized with CHAPS and then immunoprecipitated (*IP*) using the anti-VSV antibody (*second*, *fourth*, and *sixth lanes*). The immunoprecipitates were analyzed by Western blotting using the anti-5-HT$_{2C}$ receptor antiserum.

vitro experiments, confirming that the S*X*V motif at the extreme C terminus of the 5-HT$_{2C}$ receptor is the critical determinant for the interaction with MUPP1.

MUPP1 Induces 5-HT$_{2C}$ Receptor Clustering in COS-7 Cells—Because the antibodies to both MUPP1 and 5-HT$_{2C}$ receptors were derived from rabbits, a c-Myc epitope-tagged version of the human 5-HT$_{2C}$ receptor was constructed. Immunofluorescence was performed with COS-7 cells transiently expressing the *N*-terminally c-Myc-tagged 5-HT$_{2C}$ receptor. Staining with an anti-c-Myc antibody revealed a random distribution of 5-HT$_{2C}$ receptors on membrane-type structures including intracellular membranes similar to the distribution described previously (11) and in neurons (12) (Fig. 4*A*). In contrast, the MUPP1-VSV protein, which was stained with an antibody raised against the PDZ3 domain of MUPP1, was homogeneously distributed throughout transiently transfected COS-7 cells (Fig. 4*B*). The specificity of the anti-MUPP1 antibody was confirmed using the following criteria: the preimmune serum did not show any labeling (data not shown); preincubation with an excess (80 μg/ml) of a peptide (GST-PDZ3) corresponding to the PDZ3 domain suppressed the immunofluorescent staining (data not shown); and a single protein with the expected molecular mass of 230 kDa was revealed on Western blots prepared from total extracts of MUPP1-expressing COS-7 cells (data not shown) or from rat choroid plexus (see Fig. 8). Coexpression of the c-Myc-tagged 5-HT$_{2C}$ receptor together with MUPP1-VSV revealed a distribution that was distinct from those observed for each of them. Both proteins were colocalized and formed many clusters (Fig. 4*C*). A similar pattern of immunoreactive receptors was obtained when the c-Myc-tagged 5-HT$_{2C}$ receptor was coexpressed with a truncated form of the rat MUPP1 protein (VSV-ΔMUPP1) containing only the last six of the 13 PDZ domains (PDZ8–13) (Fig. 4*D*). When cells coexpressing MUPP1 and 5-HT$_{2C}$ receptors were treated with 5-hydroxytryptamine (30 min, 1 h, and overnight), no changes in the distribution of 5-HT$_{2C}$ receptors and MUPP1 were observed (data not shown).

Confocal microscopy revealed that the clustered complex of MUPP1 and 5-HT$_{2C}$ receptors was localized on intracellular membranes, but also on the cell surface (Fig. 4*E*). To further investigate cell-surface localization of the clustered complex, selective permeabilization was applied. In non-permeabilized cells, cell-surface expression of the N-terminally c-Myc-tagged 5-HT$_{2C}$ receptor could be immunostained by the anti-c-Myc antibody. When tagged 5-HT$_{2C}$ receptors were expressed alone, no significant labeling could be detected (data not shown). However, when cells coexpressed tagged receptors and MUPP1, few clusters were observed at the cell surface of non-permeabilized cells (Fig. 4*F*, *left panel*). This difference could be due to a better visualization of clustered *versus* dispersed receptors. Subsequently, the same cells were permeabilized and incubated with the anti-MUPP1 antibody, which stained numerous clusters (Fig. 4*F*, *center panel*). As expected, only those clusters localized on cell membranes were colocalized with 5-HT$_{2C}$ receptors (Fig. 4*F*, *right panel*). To investigate whether MUPP1 modulates cell-surface expression of 5-HT$_{2C}$ receptors, a cell-surface enzyme-linked immunosorbent assay was performed using non-permeabilized cells. Immunolabeled c-Myc-tagged 5-HT$_{2C}$ receptors were quantified by a secondary horseradish peroxidase-conjugated anti-mouse antibody. When c-Myc-tagged 5-HT$_{2C}$ receptors were transfected alone, the enzymatic activity was 223.45 ± 2.31% of the control values (mock-transfected cells), whereas the values were 212.95 ± 5.45% of the control values when cotransfected with MUPP1. This suggests that MUPP1 does not alter cell-surface expression of 5-HT$_{2C}$ receptors. In conclusion, these results indicate that MUPP1 induces clustering of a few 5-HT$_{2C}$ receptors at the cell surface, but the total number of cell-surface receptors remains unchanged. To test if this clustering is mediated by the C-terminal interaction of the 5-HT$_{2C}$ receptor with MUPP1, the mutant 5-HT$_{2C\text{-}SSA}$ receptor was coexpressed with MUPP1. Whereas the mutant receptor displayed a distribution similar to that of the wild-type 5-HT$_{2C}$ receptor in transfected COS-7 cells (data not shown), coexpression failed to form clusters (Fig. 4*G*).

5-HT$_{2C}$ Receptors Induce a Conformational Change in MUPP1—When MUPP1-VSV was transiently expressed in COS-7 cells, the anti-VSV antibody was unable to detect the MUPP1-VSV protein (Fig. 5*A*). However, when MUPP1-VSV was coexpressed with the 5-HT$_{2C}$ receptor, the same anti-VSV antibody succeeded in staining the MUPP1 protein (Fig. 5*B*). This clearly demonstrates that the VSV tag fused to the C terminus of the MUPP1 molecule is unmasked upon interaction of MUPP1 with the 5-HT$_{2C}$ receptor C terminus. This interaction may induce a conformational change and render the VSV tag accessible to the anti-VSV antibody.

Spatial Distribution of MUPP1 Transcripts in Rat Brain and Colocalization of MUPP1-5-HT$_{2C}$ Receptor Proteins in the Choroid Plexus—To verify that the MUPP1-5-HT$_{2C}$ receptor interaction does exist within a tissue that endogenously expresses both proteins, we first studied the spatial distribution of *MUPP1* transcripts in brain, where the distribution of 5-HT$_{2C}$ receptors is well known (11, 12). *In situ* hybridization studies were carried out using an [α-^{33}P]UTP-labeled riboprobe (Fig. 6). *MUPP1* transcripts are abundant in all cerebral cortical layers, especially the piriform cortex, the pyramidal cells of the CA1–CA3 subfields of the hippocampus, as well as the granular layer of the dentate gyrus. *MUPP1* mRNA was detected in the internal granular layer and the mitral cell layer of the olfactory bulb; in the medial habenular nucleus; and in amygdaloid,

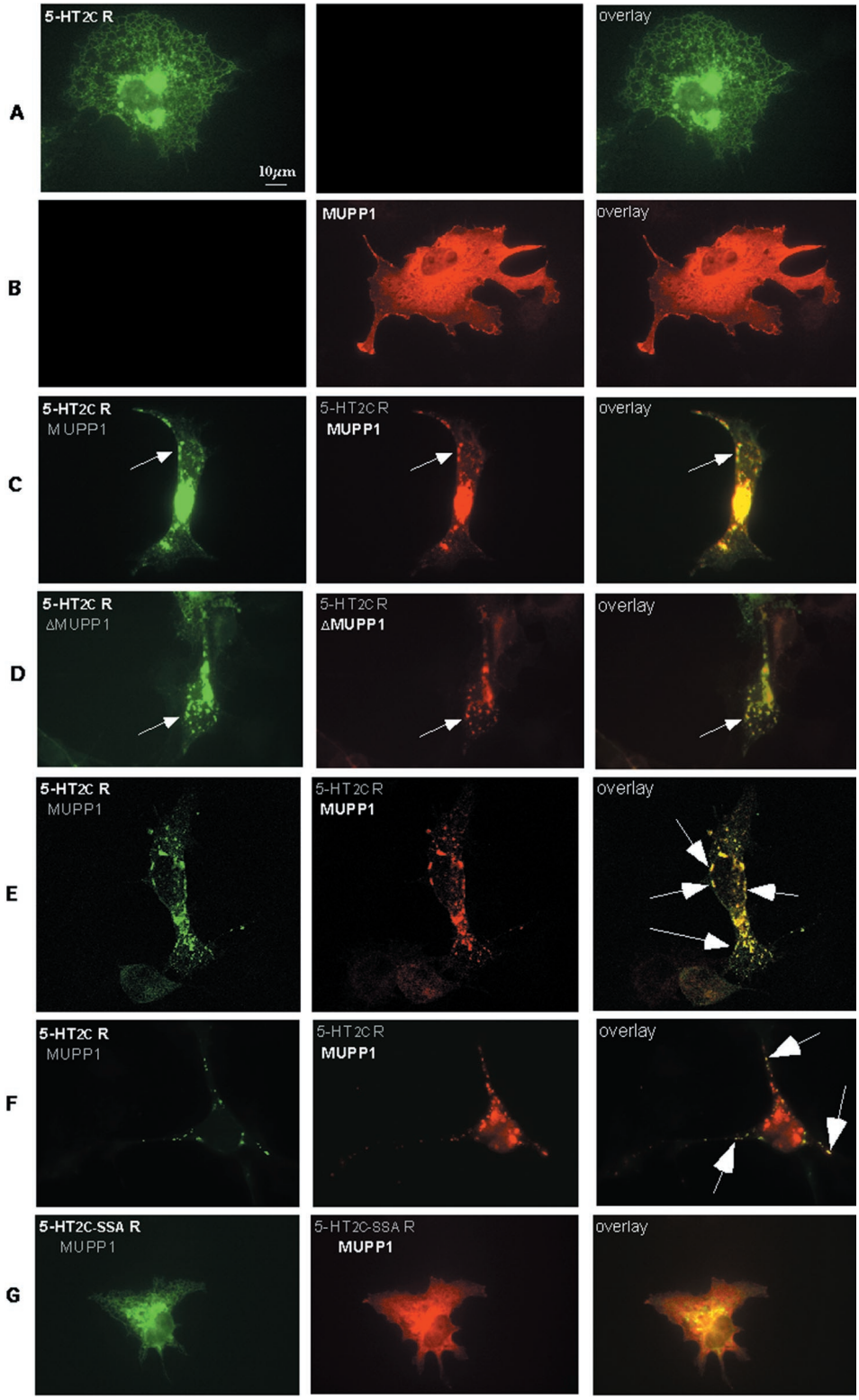


FIG. 4. **MUPP1 induces 5-HT$_{2C}$ receptor clustering in transiently transfected COS-7 cells.** *A*, cells expressing c-Myc-tagged 5-HT$_{2C}$ receptors (*5-HT2C R*) were immunostained using the anti-c-Myc antibody. *B*, cells expressing MUPP1-VSV were immunostained using the anti-MUPP1 antiserum. *C*, in cells expressing c-Myc-tagged 5-HT$_{2C}$ receptors and MUPP1-VSV, both proteins were contained within the same clusters, as indicated by *arrows*. *D*, in cells expressing 5-HT$_{2C}$ receptors and VSV-ΔMUPP1, the same clusters formed as well. *E*, shown is a confocal section of a cell expressing c-Myc-tagged 5-HT$_{2C}$ receptors and MUPP1-VSV. Most clusters were formed intracellularly. *Arrows* indicate clusters at or near the cell-surface membrane. *F*, cells expressing c-Myc-tagged 5-HT$_{2C}$ receptors and MUPP1-VSV were fixed and stained with the anti-N-terminal c-Myc antibody to label surface 5-HT$_{2C}$ receptors (*left panel*). The same cells was then permeabilized and immunostained using the anti-MUPP1 antiserum (*center panel*). *Arrows* indicate clusters at the cell-surface membrane. *G*, cells coexpressing the mutant 5-HT$_{2C\text{-}SSA}$ receptor and MUPP1-VSV failed to form clusters.

thalamic, hypothalamic, and pontine nuclei. In the cerebellum, high levels of transcripts were found in the granular layer. Transcripts were detected in the lateral ventricle, which was due to staining of the epithelial ependymal cells as well as the choroid plexus. These results indicate that *MUPP1* mRNA colocalizes with 5-HT$_{2C}$ receptor expression in all regions of the

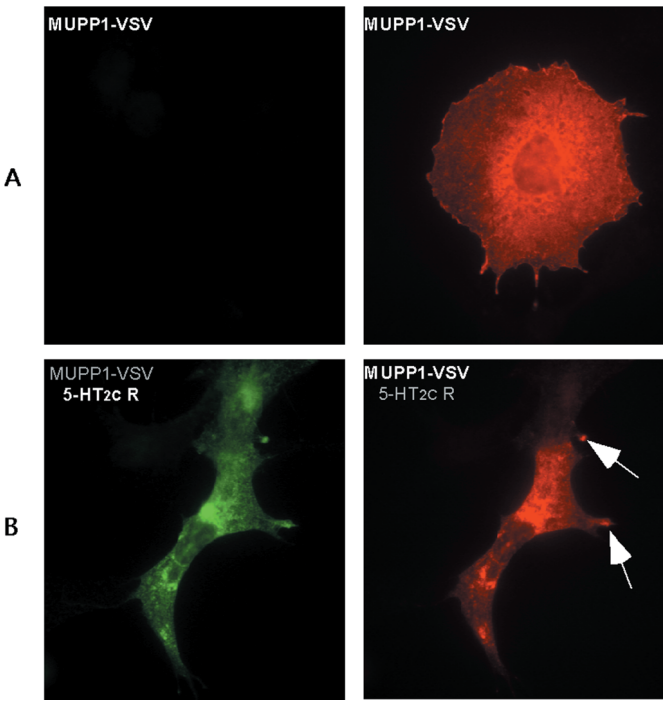


FIG. 5. **5-HT$_{2C}$ receptors unmask the VSV tag of MUPP1-VSV.** *A*, COS-7 cells were transiently transfected with MUPP1-VSV. MUPP1 was detected with the anti-MUPP1 antiserum (*right panel*), but not with the anti-VSV antibody (*left panel*). *B*, in cells expressing 5-HT$_{2C}$ receptors (*5-HT2C R*) and MUPP1-VSV, 5-HT$_{2C}$ receptors were immunostained using the anti-5-HT$_{2C}$ receptor antiserum (*left panel*), and MUPP1 was immunostained using the anti-VSV antibody (*right panel*). 5-HT$_{2C}$ receptors and MUPP1 were contained within the same clusters, indicated by *arrows*.

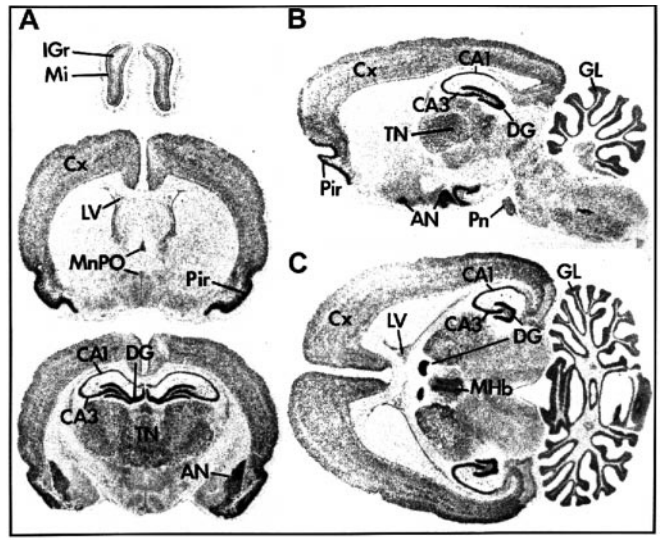


FIG. 6. ***In situ* hybridization analysis of *MUPP1* transcripts in rat brain.** Tissue sections were hybridized to ^{33}P-labeled antisense probes. Shown are representative autoradiograms of coronal sections sliced at bregma 6.7, 0.4, and 3.14 mm, respectively. *A*, sagittal section sliced laterally at 1.9 mm; *B*, a horizontal section at bregma 4.28 nm; *C*, according to the atlas of Ref. 54. Only very weak hybridization signals were observed in the granular layer of the cerebellum with the radiolabeled sense probe (data not shown). *AN*, amygdaloid nuclei; *Cx*, cortex; fields *CA1–CA3* of hippocampus; *DG*, dentate gyrus; *GL*, granular layer; *IGr*, internal granular layer of the olfactory bulb; *LV*, lateral ventricle; *Mi*, mitral cell layer of the olfactory bulb; *MHb*, medial habenular nucleus; *MnPO*, median preoptic nucleus; *Pir*, piriform cortex; *Pn*, pontine nuclei; *TN*, thalamic nuclei.

rat brain (11), including the choroid plexus, where 5-HT$_{2C}$ receptors are highly enriched (38, 39). Immunocytochemistry and confocal microscopy were applied to investigate the colocalization of MUPP1 and 5-HT$_{2C}$ receptor proteins in choroid plexus tissues. Both proteins were expressed on the apical membrane of epithelial choroid plexus cells (Fig. 7, *A* and *B*).

To demonstrate that 5-HT$_{2C}$ receptors and MUPP1 also interact *in vivo*, immunoprecipitation was performed with CHAPS-soluble extracts of rat choroid plexus. Western blot analysis revealed the presence of the MUPP1 protein in the choroid plexus (Fig. 8, *first lane*), which could also be immunoprecipitated with the anti-MUPP1 antiserum directed against the PDZ3 domain (*second lane*). The preimmune serum failed to immunoprecipitate the MUPP1 protein (Fig. 8, *fourth lane*). The anti-5-HT$_{2C}$ receptor antiserum was able to co-immunoprecipitate the MUPP1 protein (Fig. 8, *third lane*), confirming that MUPP1 and the 5-HT$_{2C}$ receptor interact in rat choroid plexus tissues.

Phosphoinositide Hydrolysis Assays—To test possible functional implications of the MUPP1-5-HT$_{2C}$ receptor interaction, serotonin-mediated phosphoinositide hydrolysis was determined in transfected COS-7 cells expressing 5-HT$_{2C}$ receptors in the absence or presence of MUPP1. In the both cases, dose-dependent stimulation of 5-HT$_{2C}$ receptors demonstrated identical EC_{50} values (0.9 ± 0.1 and 0.6 ± 0.1 nM, respectively), with an approximate 4–5-fold increase in basal inositol phosphate accumulation (Fig. 9*A*). The 5-HT$_{2C}$ receptor density as determined by Scatchard analysis of radioligand-saturation binding experiments was on the same order for transfected COS-7 cells in absence or presence of MUPP1 (Fig. 9*B*). These data indicate that the interaction of heterologously expressed 5-HT$_{2C}$ receptors with MUPP1 does not influence receptor expression levels or the second messenger activity of phosphoinositide-mediated phospholipase C activation.

DISCUSSION

The results presented here demonstrate that MUPP1 selectively interacts with the C-terminal S*X*V motif of the 5-HT$_{2C}$ receptor via PDZ10 *in vitro* and *in vivo*. Using a random tagged fragment library of MUPP1, we showed in yeast that the C terminus of the 5-HT$_{2C}$ receptor selects exclusively PDZ10 for MUPP1 interaction. This interaction requires the complete PDZ10 domain. In agreement with the literature, the 5-HT$_{2C}$ receptor terminating with a serine at position −2 selects the PDZ10 domain, which displays a histidine at the αB1 position (20). His$^{\alpha B1}$ has been shown to coordinate the hydroxyl group of the serine at position −2 of the PDZ-binding motif (40).

Immunoprecipitation of 5-HT$_{2C}$ receptors with CHAPS-soluble extracts of transfected COS-7 cells revealed glycosylated and unglycosylated receptors, which could be co-immunoprecipitated with anti-MUPP1 antibodies. This suggests that MUPP1 interacts with the glycosylated membrane-bound receptor as well as with the unglycosylated intracellular receptor reserve. Immunofluorescence in transfected COS-7 cells revealed a different localization of MUPP1 and 5-HT$_{2C}$ receptors when both were transfected separately. MUPP1 was homogeneously distributed, whereas 5-HT$_{2C}$ receptors localized within membrane-type structures, including intracellular membranes. Coexpression induced the formation of MUPP1 clusters that were strikingly different from the distribution observed with MUPP1 only. This suggests that the clustering within membranous structures was mediated by the presence of 5-HT$_{2C}$ receptors. Concerning the expression at the cell surface, MUPP1 appears to induce clustering of a few 5-HT$_{2C}$ receptors, but the total number of cell-surface receptors remains unchanged. The direct physical interaction of MUPP1 with the PDZ-binding motif of 5-HT$_{2C}$ receptors is manifested by the conformational change triggered within the MUPP1

molecule. The functional importance of the 5-HT$_{2C}$ receptor interaction with MUPP1 remains to be answered since MUPP1 did not influence receptor expression levels or the activation of phospholipase C. However, these experiments were performed in COS-7 cells, which might resemble an artificial environment. Since MUPP1 contains 13 different PDZ domains (20), a maximum of 13 different players have to be taken into account that could potentially link the MUPP1 molecule to specialized submembranous sites, thereby selectively assembling unique signaling complexes. These proteins are unlikely to be completely present in COS-7 cells. Therefore, to investigate the functional relevance of the MUPP1-5-HT$_{2C}$ receptor interaction, tissues that endogenously express both proteins have to be analyzed. Indeed, using confocal microscopy, we demonstrated that MUPP1 and 5-HT$_{2C}$ receptors colocalize exclusively at the apical surface of epithelial cells from choroid plexus tissues. The apical localization is in agreement with an early report suggesting an activation of 5-HT$_{2C}$ receptors by cerebrospinal fluid-borne serotonin (41).

Human 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors share the C-terminal E*X*(V/I)S*X*V sequence with 5-HT$_{2C}$ receptors even though all three 5-HT$_2$ receptors differ greatly in the overall identity of their intracellular C-terminal amino acid sequences. In accordance with the striking conservation of a common PDZ-binding motif at their extreme C terminus, all three receptors bind to MUPP1 PDZ domains *in vitro*. Since we demonstrated the *in vivo* interaction of MUPP1 with the 5-HT$_{2C}$ receptor, interactions with 5-HT$_{2A}$ or 5-HT$_{2B}$ receptors may occur at least in tissues displaying overlapping expression with MUPP1. In contrast to 5-HT$_{2C}$ receptors, which appear to be exclusively expressed in neuronal tissues (42), 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors are also present in peripheral organs. 5-HT$_{2A}$ receptor mRNA has been shown to be present in various human smooth muscle cells (26), and 5-HT$_{2B}$ receptor mRNA in human heart, placenta, liver, kidney, and pancreas (43, 44), tissues that are known to express MUPP1 (20). In rat brain, MUPP1 expression coincides with 5-HT$_{2A}$ transcripts in the cerebral cortex, the

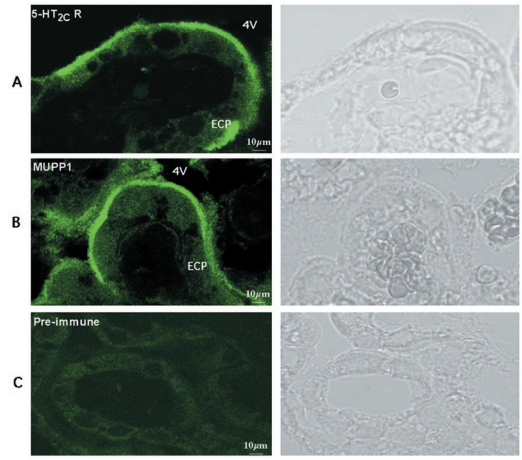
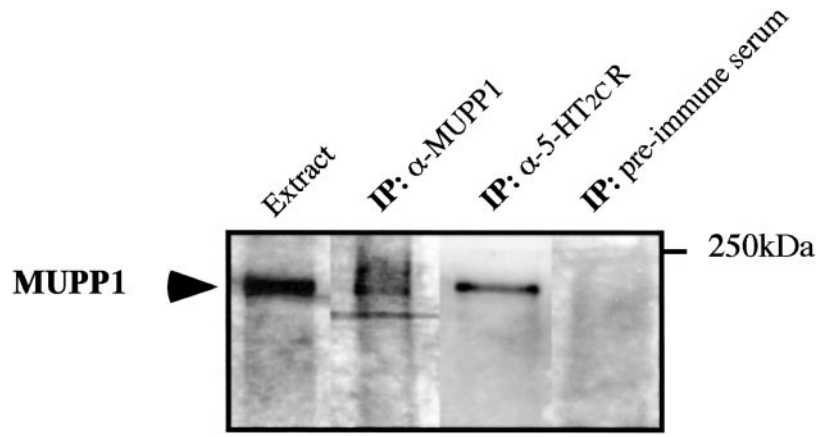


FIG. 7. **Immunohistochemistry of MUPP1 and the 5-HT$_{2C}$ receptor in the choroid plexus.** *A*, immunolabeling of a choroid plexus slice (10 μm) with the anti-5-HT$_{2C}$ receptor (*5-HT$_{2C}$ R*) antibody; *B*, immunolabeling with the anti-MUPP1 antiserum; *C*, immunolabeling with the preimmune antiserum. *4V*, fourth ventricle; *ECP*, epithelial choroid plexus cells.

FIG. 8. **Co-immunoprecipitation of MUPP1 and 5-HT$_{2C}$ receptors in CHAPS-soluble extracts of the choroid plexus.** Membrane fractions of rat choroid plexus were solubilized with CHAPS (*first lane*) and immunoprecipitated (*IP*) with the anti-5-HT$_{2C}$ receptor antiserum (*second lane*) or with the anti-MUPP1 antiserum (*third lane*). The immunoprecipitates were analyzed by Western blotting using the anti-MUPP1 antiserum. MUPP1 was not immunoprecipitated by the preimmune serum (*fourth lane*).



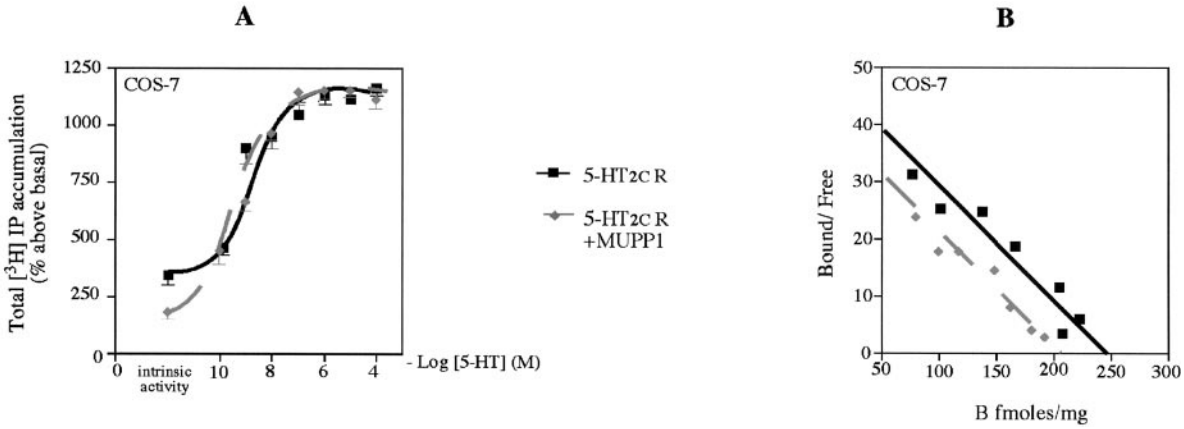


FIG. 9. **Inositol phosphate accumulation and receptor density in the absence and presence of MUPP1.** *A*, stimulation of inositol phosphate (*IP*) accumulation by increasing concentrations of 5-hydroxytryptamine; *B*, Scatchard analysis of saturation experiments of [^{3}H]mesulergine binding to membranes in COS-7 cells transfected with 5-HT$_{2C}$ receptors (*5-HT2C R*) and MUPP1 or with 5-HT$_{2C}$ receptors alone. The results are representative of three experiments performed in triplicate.

olfactory system including the mitral cell layer and the piriform cortex, the CA3 pyramidal cell layer, and the pontine nuclei (45). 5-HT$_{2B}$ receptors are coexpressed with *MUPP1* transcripts in the medial amygdala, dorsal hypothalamus, frontal cortex, and granular layer of the cerebellum (46, 47).

This is the first report of a protein interacting with a serotonin receptor. The presence of a PDZ-binding motif at the C termini of the 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors has been documented (18, 20, 48). To date, there are two reports about possible functions of the C-terminal PDZ-binding motifs of the mouse 5-HT$_{2B}$ (48) and rat 5-HT$_{2C}$ (49) receptors. The serine at position −2 was shown to enhance resensitization of the 5-HT$_{2C}$ receptor responses (49). This critical Ser^{-2} is part of two possible phosphorylation sites contained within the 5-HT$_{2C}$ receptor PDZ-binding motif. The presence of these phosphorylation sites implies that phosphorylation might regulate the capacity of the binding of the PDZ recognition sequence to the MUPP1 PDZ10 domain. Phosphorylation sites for Ser^{-2} within PDZ-binding motifs were also observed for the C terminus of the β_2-adrenergic receptor (50). It has been suggested that G-protein-coupled-receptor kinase-5-mediated phosphorylation may disrupt the interaction with NHERF and thereby regulate the sorting of internalized β_2-adrenergic receptors (4). A similar role for the PDZ-binding motif of the 5-HT$_{2C}$ receptor can be anticipated.

In a murine cell line expressing 5-HT$_{2B}$ receptors, it was demonstrated that stimulation of 5-HT$_{2B}$ receptors triggers an increase in intracellular cGMP through dual activation of constitutive and inducible nitric-oxide synthase. This activity is dependent on the C-terminal PDZ-binding motif (48). 5-HT$_{2C}$ receptors have also been shown to be involved in nitric oxide signaling such as inhibition of the *N*-methyl-D-aspartate/nitric oxide/cGMP pathway in the rat cerebellum (51) and stimulation of cGMP formation in the choroid plexus (52), which in turn inhibits phosphoinositide turnover in the choroid plexus (53). PDZ interactions with MUPP1 could provide the basis for 5-HT$_{2C}$ receptor-mediated cross-talks between second messenger pathways such as phosphoinositide hydrolysis and nitric oxide signaling. By searching for the proteins that interact with the remaining 12 PDZ domains of MUPP1, novel intracellular targets that might contribute to our understanding of 5-HT$_{2C}$ receptor trafficking and/or signaling will be identified.

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Interaction of Serotonin 5-Hydroxytryptamine Type 2C Receptors with PDZ10 of the Multi-PDZ Domain Protein MUPP1

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