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 SSV 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 SSV 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 EX/TV(SV)XV 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 assemblies 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 $\beta_2$-adrenergic receptor, which interacts with the Na$^+$/H$^+$ exchanger regulatory factor (NHERF/EBP50) (1). The interaction of NHERF with the $\beta_2$-adrenergic receptor is mediated by binding of the first PDZ domain of NHERF to the extreme C terminus of the $\beta_2$-adrenergic receptor in an agonist-dependent manner, thereby regulating Na$^+$ 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 $\beta_2$-adrenergic receptor with NHERF family proteins has been shown to control recycling of internalized $\beta_2$-adrenergic receptors. Disrupting the $\beta_2$-adrenergic receptor-NHERF interaction perturbs the endocytic sorting of the $\beta_2$-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 (sat). The subtype sst2 interacts selectively with a highly homologous PDZ domain contained within the protein CortBPI/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), a protein with 13 PDZ domains (20). MUPP1 belongs to a common family recently termed Shanks (7, 8), 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/SXV* 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/SXV* 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 CIP (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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The abbreviations used are: NHERF, Na$^+$/H$^+$ exchanger regulatory factor; 5-HT$_{2a}$, 5-hydroxytryptamine type 2C; PCR, polymerase chain reaction; GST, glutathione S-transferase; PBS, phosphate-buffered sa-

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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<sub>2C</sub> receptor interaction with MUPP1 takes place in vitro and in vivo. Among the 13 PDZ domains of MUPP1, 5-HT<sub>2C</sub> 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 domains (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). Expression Plasmids—pBSKII-rMUPP1 was cut with EcoRI and Xhol and ligated with a PCR product containing a C-terminal V5 tag flanked by two XhoI sites into pXMDI (28), generating pXMDr/rMUPP-V5. pBSKII-rMUPP1 was cut with NdeI and SalI and ligated into pCl-neo (Promega) EcoRI and SalI sites using adapter oligonucleotides (25). The V5 tag was verified using the V5 tag (YTDIEMRNLKG) DNA sequence (TACAGGATATCAGGATGAACCGCGGGAAAGTGA). The resulting plasmid, pCl-neo-V5MUPP1, fuses the N-terminal V5 tag and amino acids 1337–2055 of the rat MUPP1 protein. pRK5/5-HT<sub>2C</sub> was directionally cloned from pXMDI/h5-HT<sub>2C</sub> (29) by releasing the CDN insert with the restriction digest enzymes EcoRI and Xhol. Using PCR, the human 5-HT<sub>2C</sub> receptor cDNA was N-terminally tagged with a stretch of nucleotides (ATGGAACAAAGCTTCTCTGAAAGACTTG) encoding a 10-amino acid epitope (EQKLISEEDL) of the human c-Myc protein (30). The amplified DNA was directionally cloned into the EcoRI and Xhol sites of pRK5, yielding pRK5-c-Myc-h5-HT<sub>2C</sub>.

**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<sub>2</sub>HPO<sub>4</sub>, 20 mM CH<sub>3</sub>CO<sub>2</sub>K, 20 mM KOH, and 26.7 mM MgSO<sub>4</sub>) with 1 μg of pRK5/h5-HT<sub>2C</sub> and 1.5 μg of pXMDr/rMUPP-V5S. The total amount of DNA was kept constant at 15 μg by filling up with pRK5 DNA. After 15 min at room temperature, 10<sup>6</sup> cells were transferred to a 0.4-cm electroporation cuvette (Bio-Rad) and pulsed using a Gene Pulser apparatus (setting at 1000 microfarads and 260 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<sub>2C</sub> receptor have been described (32). The mouse monoclonal anti-c-Myc antibody was a gift from B. Mouillac. Rabbit polyclonal anti-MUPP1 anti-serum 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).<sup>2</sup> The mouse monoclonal anti-V5 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 chondroid plexus or COS-7 cell membranes were collected by centrifugation (3 min at 200 × g), and pellets were resuspended in lysis buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 0.5 mM PMSF, 10% glycerol, and 0.1% Nonidet P-40) and pelleted (100,000 × g for 1 h). Each membrane pellet was resuspended in CHAPS extraction buffer (50 mM Tris-Cl (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<sub>2C</sub> receptor 522 antibody, anti-V5 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<sub>2C</sub> receptor antibody or anti-MUPP1 antiserum (1:5000), and then detected by enhanced chemiluminescence method (Enhanced Chemiluminescence Reagent, Amersham Life Science). Total inositol phosphate accumulation in

<sup>2</sup> S. Poliak and E. Peles, manuscript in preparation.
response to serotonin in the presence of 10 mM LiCl for 10 min at 37 °C was determined as described (33). The amount of [3H]inositol phosphate formed was separated according to the ion exchange method (34). Concentration-response curves, EC50 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-HT2C receptor densities were estimated using the specific radioligand [3H]-mesulergine at a saturating concentration of 4 nM. Membranes were washed and incubated for 1 h at room temperature with the primary antibody diluted 1:500 in 0.2% gelatin and PBS. Cells were washed in 0.2% gelatin and PBS and incubated overnight at 4 °C (pH 7.4), and permeabilized with 0.05% Triton X-100 for 5 min. Cells 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 on 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-32P]UTP-labeled riboprobes that were prepared as follows. The plasmid pXMD1/rMUPP1 (20) was cut with SmaI 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 SrfI site of PCR-Script. For in vitro transcription, both sense and antisense constructs were linearized with XhoI 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-32P]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 NaHCO3 and 30 mM NaNO3, for 20 min and neutralized in 3.5 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-HT2C 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 pMN146. Since rat MUPP1 is known to interact with PDZ domains (18, 19). The 5-HT2C 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-HT2C receptor are important for binding to PDZ20 domain of MUPP1, mutational analysis was performed in which the res-
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 Ser456 and Val458 with Ala abolished the interaction with MUPP1 (Table I). In contrast, the conservative mutation of Ser456 to Thr and the mutation of Ser457 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-HT2C receptor C terminus, fusion proteins of either the wild-type form or various mutants of C-terminal 5-HT2C receptor sequences (90 amino acids) fused to GST were bound to glutathione-Sepharose (Fig. 2A) and incubated with in vitro translated 35S-labeled MUPP1 (clone 1, PDZ10–13). MUPP1 bound to the GST-5-HT2C fusion proteins was resolved on an SDS-polyacrylamide gel and visualized by autoradiography. Only the S457A mutant (Fig. 2A, fifth lane) retained MUPP1-binding activity, as observed in the yeast two-hybrid assay (Table I), which confirmed the SXV motif as a critical determinant for the PDZ domain interaction.

Interaction of MUPP1 with 5-HT2A and 5-HT2B Receptors—Multiple sequence alignment of the C-terminal ends from all members of the 5-HT2 receptor family (Fig. 2B) revealed a common C-terminal amino acid sequence motif, EX(V/I)SXV*. We therefore analyzed whether the 5-HT2A or 5-HT2B receptor C terminus would also interact with MUPP1. GST fusion proteins of the C-terminal 92 amino acids of the 5-HT2A receptor (GST-2A92) and the C-terminal 67 amino acids of the 5-HT2B receptor (GST-2B67) were bound to glutatione-coupled Sepharose beads and incubated with in vitro translated 35S-labeled MUPP1 (clone 1, PDZ10–13). MUPP1 bound to both the GST-2A92 (Fig. 2C, 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-HT2C receptor C terminus, which prevented MUPP1 binding to all three GST-5-HT2C receptor fusion proteins (Fig. 2C, 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 −9) and Val (position 0) for PDZ domain interaction.

Interaction of Heterologously Expressed 5-HT2C Receptors with MUPP1—To determine whether MUPP1 forms a protein complex with the 5-HT2C receptor in living cells, COS-7 cells were transfected with cDNA encoding the human 5-HT2C 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-HT2C receptor 522 antibody (11) revealed two bands between 60 and 50 kDa, corresponding to the glycosylated and unglycosylated 5-HT2C 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-HT2C receptor antibody. The mutant 5-HT2C receptor construct in which the C-terminal Val of the S456A and V458A mutants did not compete with MUPP1—

TABLE I

<table>
<thead>
<tr>
<th>5-HT2C carboxyl terminus (369-458)</th>
<th>HIS3</th>
<th>β-Gal</th>
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<tbody>
<tr>
<td>369-SSV (wild-type)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>369-TSV</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>369-ASV</td>
<td>–</td>
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<tr>
<td>369-SAV</td>
<td>+</td>
<td>+</td>
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<td>369-SSA</td>
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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 35S-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-HT2C 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-HT2A, 5-HT2B, and 5-HT2C receptors. All sequences share the C-terminal EX(V/I)SXV* 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-HT2A receptor (GST-2A92) or the C-terminal 67 amino acids of the 5-HT2B receptor (GST-2B67) or GST-2C90. Where indicated, an 8.5-fold excess of the EX(V/I)SXV sequence-containing peptide was present during incubation.
virus experiments, confirming that the SXV motif at the extreme C terminus of the 5-HT<sub>2C</sub> receptor is the critical determinant for the interaction with MUPP1.

**MUPP1 Induces 5-HT<sub>2C</sub> Receptor Clustering in COS-7 Cells**—Because the antibodies to both MUPP1 and 5-HT<sub>2C</sub> receptors were derived from rabbits, a c-Myc epitope-tagged version of the human 5-HT<sub>2C</sub> receptor was constructed. Immunofluorescence was performed with COS-7 cells transiently expressing the N-terminally c-Myc-tagged 5-HT<sub>2C</sub> receptor. Staining with an anti-c-Myc antibody revealed a random distribution of 5-HT<sub>2C</sub> receptors on membrane-type structures including intracellular membranes similar to the distribution described previously (11) and in neurons (12) (Fig. 4A). 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. 4B). 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<sub>2C</sub> 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. 4C). A similar pattern of immunoreactive receptors was obtained when the c-Myc-tagged 5-HT<sub>2C</sub> 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. 4D). When cells coexpressing MUPP1 and 5-HT<sub>2C</sub> receptors were treated with 5-hydroxytryptamine (30 min, 1 h, and overnight), no changes in the distribution of 5-HT<sub>2C</sub> receptors and MUPP1 were observed (data not shown).

Confocal microscopy revealed that the clustered complex of MUPP1 and 5-HT<sub>2C</sub> receptors was localized on intracellular membranes, but also on the cell surface (Fig. 4E). 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<sub>2C</sub> receptor could be immunostained by the anti-c-Myc antibody. When tagged 5-HT<sub>2C</sub> 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. 4F, 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. 4F, center panel). As expected, only those clusters localized on cell membranes were colocalized with 5-HT<sub>2C</sub> receptors (Fig. 4F, right panel). To investigate whether MUPP1 modulates cell-surface expression of 5-HT<sub>2C</sub> receptors, a cell-surface enzyme-linked immunosorben assay was performed using non-permeabilized cells. Immunolabeled c-Myc-tagged 5-HT<sub>2C</sub> receptors were quantified by a secondary horse-radish peroxidase-conjugated anti-mouse antibody. When c-Myc-tagged 5-HT<sub>2C</sub> 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<sub>2C</sub> receptors. In conclusion, these results indicate that MUPP1 induces clustering of a few 5-HT<sub>2C</sub> 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<sub>2C</sub> receptor with MUPP1, the mutant 5-HT<sub>2C</sub>-SSA receptor was coexpressed with MUPP1. Whereas the mutant receptor displayed a distribution similar to that of the wild-type 5-HT<sub>2C</sub> receptor in transfected COS-7 cells (data not shown), coexpression failed to form clusters (Fig. 4G).

**5-HT<sub>2C</sub> 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. 5A). However, when MUPP1-VSV was coexpressed with the 5-HT<sub>2C</sub> receptor, the same anti-VSV antibody succeeded in staining the MUPP1 protein (Fig. 5B). 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<sub>2C</sub> 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<sub>2C</sub> Receptor Proteins in the Choroid Plexus**—To verify that the MUPP1-5-HT<sub>2C</sub> 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<sub>2C</sub> receptors is well known (11, 12). In situ hybridization studies were carried out using an [α-<sup>35</sup>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,
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 brain.

**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-HT$_{2C}$ R) were immunostained using the anti-c-Myc antibody. B, cells expressing MUPP1-VSV were immunostained using the anti-MUPP1 antisera. 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 antisera (center panel). Arrows indicate clusters at the cell-surface membrane. G, cells coexpressing the mutant 5-HT$_{2C}$-SSA receptor and MUPP1-VSV failed to form clusters.
rat brain (11), including the choroid plexus, where 5-HT2C receptors are highly enriched (38, 39). Immunocytochemistry and confocal microscopy were applied to investigate the colocalization of MUPP1 and 5-HT2C 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-HT2C 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-HT2C receptor antiserum was able to co-immunoprecipitate the MUPP1 protein (Fig. 8, third lane), confirming that MUPP1 and the 5-HT2C receptor interact in rat choroid plexus tissues.

**Phosphoinositide Hydrolysis Assays**—To test possible functional implications of the MUPP1-5-HT2C receptor interaction, serotonin-mediated phosphoinositide hydrolysis was determined in transfected COS-7 cells expressing 5-HT2C receptors in the absence or presence of MUPP1. In the both cases, dose-dependent stimulation of 5-HT2C receptors demonstrated identical EC50 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. 9A). The 5-HT2C 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. 9B). These data indicate that the interaction of heterologously expressed 5-HT2C 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 SXV motif of the 5-HT2C 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-HT2C receptor selects exclusively PDZ10 for MUPP1 interaction. This interaction requires the complete PDZ10 domain. In agreement with the literature, the 5-HT2C receptor terminating with a serine at position −2 selects the PDZ10 domain, which displays a histidine at the o1B position (20). His481 has been shown to coordinate the hydroxyl group of the serine at position −2 of the PDZ-binding motif (40).

Immunoprecipitation of 5-HT2C 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-HT2C receptors, but the total number of cell-surface receptors remains unchanged. The direct physical interaction of MUPP1 with the PDZ-binding motif of 5-HT2C receptors is manifested by the conformational change triggered within the MUPP1...
molecule. The functional importance of the 5-HT\textsubscript{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\textsubscript{2C} receptor interaction, tissues that endogenously express both proteins have to be analyzed. Indeed, using confocal microscopy, we demonstrated that MUPP1 and 5-HT\textsubscript{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\textsubscript{2C} receptors by cerebrospinal fluid-borne serotonin (41).

Human 5-HT\textsubscript{2A} and 5-HT\textsubscript{2B} receptors share the C-terminal EXV/DSXV sequence with 5-HT\textsubscript{2C} receptors even though all three 5-HT\textsubscript{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 \textit{in vitro}. Since we demonstrated the \textit{in vivo} interaction of MUPP1 with the 5-HT\textsubscript{2C} receptor, interactions with 5-HT\textsubscript{2A} or 5-HT\textsubscript{2B} receptors may occur at least in tissues displaying overlapping expression with MUPP1. In contrast to 5-HT\textsubscript{2C} receptors, which appear to be exclusively expressed in neuronal tissues (42), 5-HT\textsubscript{2A} and 5-HT\textsubscript{2B} receptors are also present in peripheral organs. 5-HT\textsubscript{2A} receptor mRNA has been shown to be present in various human smooth muscle cells (26), and 5-HT\textsubscript{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\textsubscript{2A} transcripts in the cerebral cortex, the
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 \(\beta_2\)-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 \(\beta_2\)-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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