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The PDZ Protein Mupp1 Promotes $G_i$ Coupling and Signaling of the Mt$_1$ Melatonin Receptor$^a$

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The concept of organized networks has emerged in the field of cellular signaling in the last few years. Assembling the different partners in close proximity optimizes the spatial and temporal organization and the specificity of the cellular response. The assembly of these multimolecular complexes occurs through the interaction of modular domains recognizing their target counterparts. PDZ domains are widely spread modules exhibiting this function. Data bank exploration with SMART (1) identifies 584 PDZ domains in 328 different proteins in the human genome.

$G$ protein-coupled receptors (GPCRs)$^5$ constitute the largest family of membrane receptors, and many of the more than 750 members have been shown to interact with PDZ domain-containing proteins, either constitutively or upon agonist activation (2). Binding of PDZ proteins to GPCRs has been reported to primarily regulate subcellular localization, trafficking, and stability of receptors (3). For instance, binding of MUPP1 and syntrophins to the $\gamma$-aminobutyric acid type B (GABA$_\beta$) receptor and the $\alpha_2$-adrenergic receptor, respectively, significantly increases receptor stability (4, 5). In other cases, PDZ scaffolds determine the subcellular localization of GPCRs (6) and receptor endocytosis as shown for PSD-95 and the $\delta$-HT$_2$A serotonin and $\beta_1$-adrenergic receptors (7, 8). PDZ proteins, such as NHERF and hScrib, are also important for the recycling of receptors to the cell surface (9–11).

Binding of PDZ proteins to GPCRs also modulates receptor signaling by assembling proteins involved in signal transduction. NHERF family proteins are known to regulate the activity of the $Na^+/H^+$ exchanger through association with NHERF-1 (12) and to form a ternary complex with phospholipase $C\beta$3 and GPCRs, which enhances the signaling efficiency of the receptor-mediated activation of the phospholipase $C\beta/Ca^{2+}$ pathway (13–15). Binding of GIPC (GAIP-interacting protein, COOH terminus) to the COOH terminus of the D3 dopamine and the $\beta_1$-adrenergic receptor (16) decreased Gz$_\alpha$-mediated signaling of these receptors most likely through RGS19, which binds to GIPC (17). Further examples of PDZ scaffolds that regulate GPCR signaling include a ternary complex formation around the PDZ scaffold MAGI-3, which binds to the GPCR frizzled-4 and Ltap to regulate the JNK signaling cascade (18), as well as PDZ-domain-containing Rho guanine nucleotide exchange factors that interact with lysophosphatidic acid 1 and 2 receptors to activate RhoA (19).

To identify proteins that specifically interact with the G protein-coupled human MT$_1$ and MT$_2$ melatonin receptors, we
performed a yeast two-hybrid screen using the cytoplasmic domains of these receptors as baits. The multi-PDZ domain protein MUPP1 was identified as interacting partner of the carboxyl-terminal tail (C-tail) of MT₁, but not of MT₂. Importantly, this interaction was necessary for the stabilization of the MT₁-Gₛ complex and efficient Gₛ-dependent signaling of MT₁.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen**—The cDNA sequences corresponding to the intracellular loops i₂ (residues 123–141), i₃ (residues 208–239), and the C-tail (residues 294–350) of MT₁ or the C-tail alone were inserted in frame in the pB6 yeast expression vector derived from the original pAS2ΔΔ (20). A random-primed, size-selected (mean insert size 800 bp) cDNA library of differentiated human brown adipocyte PAZ6 cells (21) was constructed in the pB6 vector derived from the original pGADGH vector. Plasmids able to rescue yeast growth were amplified by PCR and sequenced at their 5’ and 3’ junctions on a PE3700 sequencer. The resulting sequences were used to identify the corresponding interactors in the GenBank™ data base (NCBI) using a fully automated procedure.

**Plasmid Constructions and Cell Culture**—The GW1-HA-MUPP1 plasmid containing the coding region of the rat MUPP1 as well as GST fusion constructs expressing PDZ1–3, PDZ4–5, PDZ6, PDZ7, or PDZ8–9 were a gift from Dr. Javier Mancini (24). GST constructs were expressed in grown and transfected as described (25).

**Solubilization and Immunoprecipitation**—Cells were lysed for 4 h on ice in lysis buffer (25 mM HEPES, 150 mM NaCl, 2 mM EDTA, 15 mM β-glycerophosphate, 2 mM Na₃VO₄, 10 mM NaF, 5 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml benzamidine, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride) containing 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS, and lysates were centrifuged at 26,000 × g for 30 min at 4 °C. Receptor immunoprecipitation was done on supernatant by the anti-FLAG M2 antibody (Sigma) preadsorbed on Protein G. Immunoadsorbed material was pelleted by centrifugation, submitted to SDS-PAGE, and transferred to nitrocellulose. Immunoblot analysis was carried out with the polyclonal anti-MUPP1 antibody at 1:20,000 dilution (a gift from Dr. Javier) (22, 23) or the clonal anti-FLAG M2 antibody and polyclonal anti-HA antibodies (27). JUNE 13, 2008 • VOLUME 283 • NUMBER 24 JOURNAL OF BIOLOGICAL CHEMISTRY 16763
cells were lysed, and the solubilized receptor concentration was adjusted to 5 nM. Dilutions of the solubilized receptor were incubated on preadsorbed GST-fused PDZ domains, and the ELISA was performed as described above.

Binding Assays—[125I]MLT (PerkinElmer Life Sciences) binding assays were performed on membranes as described (29).

Receptor Internalization—For constitutive internalization, suspended HEK-FLAG-MT1 cells were incubated with anti-FLAG M2 antibody (1 h, 4 °C). Aliquots were then incubated at 37 °C for variable times. Cells were then transferred to ice, incubated with fluorescein isothiocyanate-coupled secondary antibody, and fixed with paraformaldehyde. The fluorescence was measured by fluorescence-activated cell sorting.

For agonist-stimulated internalization, cells in 6-well plates were incubated in culture medium containing 0.1 μM MLT for variable times (0.5–3 h) and then transferred to ice. Cells were suspended and incubated with anti-FLAG M2 antibody, followed by fluorescein isothiocyanate-coupled secondary antibody to label surface receptor. Fluorescence was measured by fluorescence-activated cell sorting after fixation with paraformaldehyde.

Receptor Degradation—HEK-FLAG-MT1 cells were plated in polylysine-coated 24-well plates. The next day, protein synthesis was inhibited by treatment for 45 min with 100 μM cycloheximide. Incubation was continued for 4 h in the presence or absence of agonist (0.1 μM MLT). Cells were transferred to ice, fixed, and permeabilized by treatment with cold ethanol at −20 °C for 10 min. ELISAs were performed as described above.

cAMP Assay—Cyclic AMP levels were determined by HTRF using the Cisbio "cAMP femto2" kit according to the manufacturer’s instructions. Samples were analyzed with a PheraStar apparatus (BGM Labtech, Offenburg, Germany).

Bioluminescence Resonance Energy Transfer (BRET) Assay, Luminescence, and Fluorescence Measurements—BRET experiments, luminescence, and fluorescence measurements were performed as described on adherent cells (25).

siRNA Treatment—siRNAs corresponding to region 955–973 of the human MUPP1 cDNA were synthesized (Eurogentech, Seraing, Belgium) and transfected with Lipofectamine 2000 (Roche Applied Science) according to the supplier’s instructions. Negative control siRNA Alexa Fluor 488 was from Qiagen (catalog number 1022563).

Mitogen-activated Protein Kinase Activation—Activated ERK1/2 were detected by anti-phospho-ERK antibody (sc-7383; Santa Cruz Biotechnology). Levels of loaded proteins were compared by detection of ERK2 (sc-154; Santa Cruz Biotechnology).

Statistical Analysis—Results were analyzed by PRISM (GraphPad Software Inc., San Diego, CA). Data are expressed as mean ± S.E. Student’s t test was applied for statistical analysis.

RESULTS

The C-tail of MT1 Specifically Interacts with MUPP1—To identify interacting partners of the human melatonin receptor MT1 and MT2 receptors, we conducted yeast two-hybrid screens using the C-tails or a fusion of the intracytoplasmic loops i2 and i3 and the C-tail of these receptors as baits (Fig. 1A). The screens were performed against a cDNA library of differentiated human brown adipocytes, a cellular context known to express functional melatonin receptors (31). Among the positive clones that interacted with the two baits containing the C-tail of MT1, we identified 14 sequences corresponding to the indicated region of human MT1, encompassing part of PDZ9 and PDZ11 and the entire PDZ10 domain. The identification of a PDZ domain-containing protein as a specific interacting partner of the C-tail of MT1 is consistent with the presence of a canonical class III PDZ domain-binding motif DSV-COO− at the COOH terminus. To further test the specificity of our yeast two-hybrid screen, we used the C-tail of the β2-adrenergic receptor as bait. Despite the presence of a functional PDZ binding motif at the C-tail of this receptor, we were unable to recover MUPP1, indicating the high specificity of the MT1/MUPP1 interaction (data not shown).

To confirm the interaction between the C-tail of MT1 and MUPP1 in a different experimental setting, we incubated MT1 or MT2 C-tails, which were chemically synthesized with a His6 tag and immobilized on beads, with whole brain lysates from mice. We then tested for the presence of MUPP1 among the retained proteins with anti-MUPP1 antibodies (Fig. 1C). MUPP1 was bound to the C-tail of MT1, but not to that of MT2, confirming the specificity of the interaction.

FIGURE 1. The C-tail of MT1 interacts with MUPP1. A, schematic representation of melatonin receptor baits (C-tail or a fusion of the i2 loop, i3 loop, and C-tail) used in the yeast two-hybrid screen. B, screening of a human brown adipocyte cDNA library with MT1 baits identified 14 different sequences corresponding to the indicated region of MUPP1. The shared amino acid sequence (*, corresponding to the indicated region of MUPP1. The sequence shared by all clones is indicated in boldface type (nucleotides 4548–5189, amino acids 1516–1729). C, solubilized brain proteins were incubated with the immobilized His6-tagged C-tail of MT1, or MT2. The presence of MUPP1 among the retained proteins was evaluated by Western blotting with anti-MUPP1 antibodies.

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Effect of PDZ10 on MT1 Internalization and Degradation

Interaction of GPCRs with PDZ proteins has been shown to stabilize receptors by inhibiting either their constitutive or agonist-promoted internalization (8, 34) or by interfering with receptor degradation (4, 5). To study the role of MUPP1 in MT1 internalization and degradation, we used the isolated PDZ10 domain of MUPP1 as a dominant negative to disrupt the MUPP1/MT1 interaction. As shown in Fig. 4A, the amount of MUPP1 associated with the receptor is, as expected, strongly decreased in the presence of PDZ10. We first determined the rate of constitutive internalization of MT1 in the absence and presence of PDZ10. The amount of endogenous MT1 receptors (33) and MUPP1 (data not shown) was significantly increased in the presence of anti-MUPP1 antibodies, demonstrating the existence of MT1/MUPP1 complexes in native tissue.

To further confirm the specificity of the interaction, we transiently expressed similar levels of FLAG-MT1, FLAG-MT1-Δcter, or HA-MUPP1 with HA-MUPP1 and WT-MT1 directly in intact cells by co-immunoprecipitation experiments in HEK-FLAG-MT1 cells. As shown in Fig. 3E, significant amounts of radiolabeled MT1 were precipitated in the presence of anti-MUPP1 as compared with irrelevant control antibodies, demonstrating the existence of MT1-MUPP1 complexes in native tissue.

Overall, our in vitro data show that MUPP1 interacts with MT1 with high affinity and that this interaction involves the PDZ10 domain of MUPP1 and the COOH-terminal DSV motif of MT1. The interaction mainly depends on the COOH-terminal valine residue, although other amino acids, such as the aspartate residue at position −2, also appear to be involved.

Interaction between MT1 and MUPP1 in Mammalian Cells

To investigate the interaction of MUPP1 with MT1 in mammalian cells, we expressed HA-tagged MUPP1 in HEK-FLAG-MT1 cells (100 fmol/mg of protein) (25). As shown by immunofluorescence staining with anti-HA antibodies, a small amount of MUPP1 was present throughout the cytoplasm, but the majority was located at the plasma membrane, where it colocalized with MT1 (Fig. 3, A–C). The interaction of both proteins was addressed in intact cells by co-immunoprecipitation experiments in HEK-FLAG-MT1 cells. As shown in Fig. 3D, anti-FLAG antibodies coprecipitated endogenous MUPP1.

To demonstrate the presence of the protein complex in native tissue, we performed immunoprecipitation studies with ovine pituitary PT tissue samples known to express significant amounts of endogenous MT1 receptors (33) and MUPP1 (data not shown). Receptors were labeled with the specific [125I]MLT radioligand, and protein complexes were solubilized and immunoprecipitated with anti-MUPP1 antibodies. As shown in Fig. 3E, significant amounts of radiolabeled MT1 were precipitated in the presence of anti-MUPP1 as compared with irrelevant control antibodies, demonstrating the existence of MT1-MUPP1 complexes in native tissue.

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Affinity and Molecular Determinants of the MT1/MUPP1 Interaction

To delineate the molecular determinants of the MT1/MUPP1 interaction in vitro, we used receptor mutants of the PDZ binding motif and GST fusion proteins of the different PDZ domains of MUPP1 (Fig. 2A) to perform GST pull-down experiments (see supplemental Fig. S1 for GST constructs). Only GST-PDZ10 was able to specifically precipitate the NH2-terminally FLAG-tagged MT1, confirming that this domain is involved in the interaction. Mutation of the very last valine residue into alanine (MT1-Δcter) completely abolished the interaction, as did the deletion of the entire C-tail (MT1-Δcter). Mutation of the aspartate residue at position −2 (MT1-ASV) reduced the amount of precipitated receptor.

To confirm these interactions in a more quantitative assay, we used an ELISA set-up where similar quantities of the GST-PDZ fusion proteins were immobilized (Fig. 2B) and incubated with equivalent quantities of solubilized receptors, as confirmed by Western blot (not shown). Only PDZ10 was able to interact with MT1. Although the MT1-ASV mutant was partially retained, MT1-Δcter and MT1-DSA completely failed to interact with PDZ10. We then determined the dissociation constant (Kd) of the MT1/PDZ10 interaction with the ELISA (Fig. 2C). Whereas binding to immobilized PDZ10 was saturable and of high affinity (Kd = 3.8 ± 0.7 nM), only background binding was detected for PDZ9-coated wells.

Overall, our in vitro data show that MUPP1 interacts with MT1 with high affinity and that this interaction involves the PDZ10 domain of MUPP1 and the COOH-terminal DSV motif of MT1. The interaction mainly depends on the COOH-terminal valine residue, although other amino acids, such as the aspartate residue at position −2, also appear to be involved.
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FIGURE 3. Interaction between MUPP1 and MT1 in mammalian cells. A–C, confocal images of HEK293 cells showing a partial localization of HA-MUPP1 and FLAG-MT1, at the plasma membrane, FLAG-MT1, and HA-MUPP1 were detected by immunofluorescence using anti-FLAG or anti-HA antibodies. D, co-immunoprecipitation (IP) of endogenous MUPP1 in HEK-FLAG-MT1 (lane 2) or HEK 293 cells (lane 1). Data are representative of at least two further experiments. E, co-immunoprecipitation of MT1, with MUPP1 in ovine pituitary pars tuberalis; [125I]MLT-labeled receptors (45 fmol/mg membrane proteins) were immunoprecipitated from solubilized membranes by anti-MUPP1 antibodies or control rabbit sera (pool of five preimmune rabbit sera). Nonspecific immunoprecipitated binding was evaluated in the presence of 1 μM MLT. F and G, lysates from HEK 293 cells expressing HA-MUPP1 alone or with FLAG-MT1, FLAG-MT1-ASV, FLAG-MT1-DSA, or FLAG-MT1-Δcter were prepared (F), receptors were immunoprecipitated, and precipitates were analyzed by Western blot (WB) for the presence of MUPP1 (G). H, time course of MLT (10 nM) stimulation in HEK-FLAG-MT1 cells transfected with HA-MUPP1. Western blot analysis of HA-MUPP1 was performed on anti-FLAG immunoprecipitates by anti-HA antibodies (G) or anti-MUPP1 antibodies (F). Data are representative of at least two further experiments.

presence of PDZ10. Fig. 4B shows that the rate of internalization is not affected in the presence of PDZ10; in addition, the MT1-DSA mutant, unable to interact with MUPP1, presents equivalent constitutive internalization characteristics. Similarly, the binding of MUPP1 to MT1 appears not to alter ligand-induced receptor internalization by 100 nM MLT, since equivalent internalization kinetics and maximal internalization of ∼60% within 3 h were observed in the absence and presence of PDZ10 (Fig. 4C) and for the MT1-DSA mutant. The degradation of MT1 was studied by treating cells with the protein synthesis inhibitor cycloheximide (100 μM) for 4 h. The more than 50% decrease in receptor number, in the absence of MLT, suggests that constitutively internalized receptors are mostly degraded. Simultaneous MLT treatment (100 nM) moderately increased MT1 degradation (Fig. 4D). Similar effects were observed for MT1-DSA. Coexpression of PDZ10 did not modify unstimulated and MLT-stimulated MT1 degradation. These results indicate that MUPP1 has no significant effect on MT1 endocytosis and degradation.

MUPP1 Is Necessary for Signaling of MT1 through the Adenylyl Cyclase Pathway—MT1 is a predominantly Gs-coupled GPCR that inhibits adenylyl cyclase (AC) activity in primary cell cultures and transfected cells (29, 35). Stimulation of HEK-
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FIGURE 5. Signaling of MT₁ through adenylyl cyclase depends on the presence of MUPP1. A, HEK-FLAG-MT₁ cells transfected with the indicated cDNAs or siRNA duplexes or FLAG-MT₁-DSA cells were stimulated with MLT (10 nM) or not in the presence of 1 μM forskolin (Fsk), and intracellular cAMP levels were determined. B, inhibition of endogenous MUPP1 expression. HEK-FLAG-MT₁ were transfected with a scrambled control siRNA (lane 1) or MUPP1-specific siRNA duplexes (lane 2). Lysates were immunoprecipitated with anti-FLAG antibody, and MUPP1 was revealed by anti-MUPP1 antibodies. C, dose-response curves of MLT-induced inhibition of forskolin-stimulated cAMP production in HEK-FLAG-MT₁ and FLAG-MT₁-DSA cells (curves indicated by 1 and 2, respectively). D, CAMP response induced by the ligand in Fsk-stimulated HEK293 cells (1 μM) transiently transfected with the G₁-coupled somatostatin receptor SSTR2 or chemokine receptor CCR5 (10 nM somatostatin or 100 nM RANTES, respectively) or the G₁-coupled β₂-adrenergic receptor (β₂AR) (1 μM isoproterenol) in the absence or presence of MUPP1 siRNA. Data are means ± S.E. of at least three independent experiments, each performed in triplicate.

MLT-MT₁ cells with a saturating MLT concentration (10 nM) resulted in the expected decrease of forskolin-stimulated cAMP levels of about 60% (Fig. 5A). Expression of the dominant negative PDZ10 in these cells strongly attenuated the inhibitory effect of MT₁ on cAMP production, indicating that binding of MUPP1 to MT₁ is necessary for efficient coupling of the receptor to the cAMP pathway. To verify that the displaced protein corresponds to MUPP1, we treated cells with MUPP1-specific siRNA molecules. MUPP1 expression was decreased by nearly 80% compared with the control siRNA (Fig. 5B), and the MLT-induced decrease in cAMP production was abolished to the same extent as in cells expressing the dominant negative PDZ10 domain (Fig. 5A). Importantly, control siRNA molecules did not interfere with the inhibitory effect of MLT on forskolin-stimulated cAMP levels. This strongly suggests that binding of MUPP1 to the MT₁ C-tail is essential for its efficient coupling to the AC pathway. To further support this idea, we tested the MT₁-DSA mutant, which is unable to interact with MUPP1. Consistently, 10 nM MLT stimulation of HEK 293 cells stably expressing MT₁-DSA receptors at similar expression levels as HEK-FLAG-MT₁ cells did not inhibit forskolin-stimulated cAMP levels (Fig. 5, A and C). To further assess the specificity of the MUPP1 interaction with MT₁ on the AC pathway, we measured the effect of MUPP1 knockdown on two other G₁-coupled receptor, the human SSTR2 somatostatin receptor and the CCR5 chemokine receptor. Indeed, MUPP1 siRNA treatment of cells transiently transfected with each of these receptors had no effect on the cAMP inhibition elicited by 10 nM somatostatin or 100 nM RANTES, respectively (Fig. 5D). In addition, the siRNA treatment had no effect on the cAMP response observed for the β₂-adrenergic receptor, a G₁-coupled receptor, stimulated by 1 μM isoproterenol (Fig. 5D). Overall, our results show that binding of MUPP1 to the COOH-terminal DSV motif of MT₁ plays a central role in MT₁ signaling through the AC pathway.

G₁ Coupling and High Affinity Agonist Binding to MT₁ Depends on the Presence of MUPP1—We hypothesized that the inability of FLAG-MT₁ to couple to the AC pathway in the absence of MUPP1 may be due to a defect in G₁ coupling to the receptor. In agreement with previous reports (29), solubilization of MT₁ under mild conditions preserved the interaction with Gα₁ proteins (Fig. 6A). In contrast, coexpression of PDZ10 strongly decreased the quantity of coprecipitated Gα₁. Consistently, in cells stably expressing the MUPP1 binding-deficient FLAG-MT₁-DSA mutant, Gα₁ proteins were undetectable under these conditions. Decreased G protein coupling was specific for Gα₁, since Gα₁q, readily precipitated under all conditions. These results show that the inability of MT₁ to signal through the AC pathway in the absence of MUPP1 is most likely due to its reduced G₁ coupling capacity.

Previous studies have shown that high affinity agonist binding to MT₁ depends on the coupling of the receptor to G₁ proteins (36). Accordingly, decreased high affinity agonist binding would be expected when MUPP1 is displaced from MT₁. We therefore incubated cell membranes prepared from HEK-FLAG-MT₁ cells in the presence or the absence of purified PDZ9 or PDZ10 and determined agonist binding using the radiolabeled MLT receptor agonist [125I]MLT (Fig. 6B). In the absence of added PDZ domain, MT₁ bound [125I]MLT with the expected high affinity (KD = 136 ± 8 pm). Similar results were obtained in the presence of PDZ9 (KD = 160 ± 15 pm). In contrast, in the presence of PDZ10, a significantly lower affinity was observed (KD = 378 ± 79 pm) (p < 0.05; MT₁ alone versus MT₁ + PDZ10). The number of binding sites was not affected in any of the conditions (Bmax = 100 ± 9, 94 ± 9, and 118 ± 13% for MT₁, MT₁ + PDZ9, and MT₁ + PDZ10, respectively). The lower affinity for [125I]MLT in the presence of PDZ10 is consistent with the lower affinity of the MT₁-DSA mutant (KD = 321 ± 50 pm), which is devoid of MUPP1 binding. These results indicate that binding of MUPP1 to the MT₁ C-tail participates in high affinity agonist binding, most likely by stabilizing G₁ binding to the agonist-activated receptor.
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To show the formation of a trimeric complex between MT₁, Gᵢ, and MUPP1, we used the BRET assay, which allows detection of real-time protein interactions in a cellular context (54). The previously described Gᵢᵢ₁₋₉₁-Rluc fusion protein (energy donor) (37) was coexpressed with a fragment of MUPP1 comprising PDZ domains 9–13 fused at its amino-terminal tail to the energy acceptor YFP (YFP-PDZ9–13) (37) was coexpressed with a fragment of MUPP1 comprising PDZ domains 9–13 fused at its amino-terminal tail to the energy acceptor YFP (YFP-PDZ9–13)

Expression of both proteins in HEK 293 cells revealed a nonspecific interaction between Gᵢ and MUPP1 (weak signal, linear and nonsaturating behavior of BRET donor saturation curve) (Fig. 6C). In contrast, when YFP-PDZ9–13 and GoᵢⅢ₉₁-Rluc were expressed in HEK-FLAG-MT₁ cells, Gᵢ interacted with high affinity with MUPP1 as shown by the hyperbolic and saturable behavior of the BRET donor saturation curve. Stimulation of cells with MLD did not alter BRET signals (not shown).

The high affinity interaction between Gᵢ and YFP-PDZ9–13 was specific, since no interaction was observed for YFP-PDZ1–4 and YFP-PDZ5–8 constructs in HEK-FLAG-MT₁ cells. To evaluate the functional importance of the PDZ9–13 subdomain on the AC pathway, we co-transfected FLAG-tagged MT₁ and PDZ9–13 in HEK293 cells devoid of MUPP1 (MUPP1 siRNA-treated). Although the MLD-promoted inhibition of Fsk-stimulated cAMP production was abolished in MUPP1 siRNA-treated cells as expected, simultaneous expression of PDZ9–13 did not allow us to reestablish the MT₁ response (Fig. 6D). Indeed, PDZ9–13 by itself inhibits the MT₁ response, as was observed for the single PDZ10 fragment. Collectively, our results indicate that the PDZ9–13 fragment of MUPP1 is necessary for high-affinity binding of Gᵢᵢ to MT₁ but not sufficient to reconstitute a functional system to modulate the AC pathway.

Disruption of MUPP1/MT₁ Interaction Affects MLD-stimulated ERK Activation—Many GPCRs activate the mitogen-activated protein kinase pathway, although through different mechanisms (38). A common mechanism of mitogen-activated protein kinase activation involves Gᵢ proteins. As shown in Fig. 7A, 10 nM MLD-promoted ERK phosphorylation was abolished in PTX-treated cells, indicating that ERK activation by MT₁ is indeed Gᵢ-dependent. We studied the kinetics of ERK phosphorylation in the presence and absence of PDZ10, to determine the effect of MUPP1 on ERK activation. In both cases, the amplitude and time course of ERK activation was similar with maximal effects at 5 min of MLD stimulation (Fig. 7, B and C). Similar EC₅₀ values were obtained for the MT₁ wild-type and the MUPP1 binding-deficient MT₁-DSA mutant (Fig. 7, C and D). These results suggest that Gᵢ-dependent ERK signaling of MT₁ is not altered in the absence of MUPP1 binding. Such an observation is in apparent contradiction to our data on the cAMP pathway. To account for the different effect of MUPP1 on both pathways, we then hypothesized that the AC pathway can be more sensitive to alterations of the Gᵢ coupling to MT₁ than the mitogen-activated protein kinase pathway. We therefore decreased the amount of functional Gᵢ proteins by incubating cells with increasing doses of PTX and determined the degree of MLD-promoted ERK phosphorylation of wild-type MT₁ or the MT₁-DSA mutant (Fig. 7E). Maximal doses of PTX inhibited ERK activation for both receptors to a similar extent. This shows that the ERK activation of the MT₁-DSA mutant also depends on Gᵢ protein activation and excludes the possibility that ERK activation becomes Gᵢ-dependent in the absence of functional Gᵢ coupling. At submaximal PTX concentrations, ERK activation by the MT₁-DSA mutant and the wild-type receptor was clearly different (IC₅₀ = 0.10 and 0.02 ng/ml for wild-type MT₁ and MT₁-DSA, respectively). The left shift of the dose-response curve of ERK phosphorylation of the MT₁-DSA mutant indicates that ERK activation becomes indeed more sensitive to the amount of active Gᵢ proteins in the absence of MUPP1 binding to MT₁. Taken together, both Gᵢ-dependent signaling pathways, the AC and the ERK pathways, are affected in the absence of MUPP1 binding to MT₁.
PDZ scaffolds have been shown to modulate GPCR signaling in different ways. Binding of GPCRs to PDZ scaffolds has been reported to modulate the amount of receptor at the cell surface and consequently the amplitude of the functional response by altering the receptor’s trafficking and stability (2). Other examples highlight the importance of the scaffolding properties of PDZ domain proteins in GPCR signaling. For instance, simultaneous binding of the PDZ scaffold GIPC to the G<sub>i</sub>-coupled D<sub>2</sub> dopamine receptor and to RGS19 favors the GDP/GTP exchange of G<sub>i</sub> by RGS19 (17). Moreover, binding of NHERF-1 to GPCRs and phospholipase C<sub>β3</sub> enhances the signaling efficiency of the phospholipase C<sub>β</sub>/Ca<sup>2+</sup> pathway (13–15). Modulation of MT<sub>1</sub> signaling by MUPP1 probably involves a previously unappreciated regulatory mechanism. Disruption of the interaction of MUPP1 with the C-tail of MT<sub>1</sub> decreased signaling of MT<sub>1</sub> through the AC and the ERK pathway. Importantly, the interaction between G<sub>i</sub> proteins and MT<sub>1</sub> was also destabilized under these conditions, suggesting that MUPP1 regulates MT<sub>1</sub> signal transduction by stabilizing the receptor-G<sub>i</sub> protein complex. This indicates physical proximity between MUPP1 and G<sub>i</sub> proteins, as supported by our BRET experiments. However, more complex mechanisms cannot be excluded, since PDZ9–13, which is able to restore high affinity binding of G<sub>i</sub> to MT<sub>1</sub>, is insufficient to reconstitute functional coupling to AC. Some PDZ proteins are indeed able to physically interact with heterotrimeric G proteins, as recently reported for PSD95, SAP97, and Vel12, which interact with G<sub>i</sub><sub>α</sub> and G<sub>i</sub><sub>β</sub> (39). Furthermore, α-syntrophin has been shown to bind to G<sub>i</sub><sub>α</sub>γ through its PDZ domain (40). Although G<sub>β</sub> subunits typically code for the “LWL” or “IWN” sequence at their C-tails, several G<sub>γ</sub> subunits (e.g. G<sub>γ</sub><sub>δ</sub>, G<sub>γ</sub><sub>12</sub>, and G<sub>γ</sub><sub>13</sub>) have the COOH-terminal “TIL” sequence, which corresponds to the class I ((S/T)XL) PDZ domain recognition sequence. Alternatively, MUPP1 and G<sub>i</sub> may be physically linked through a third protein. According to our BRET experiments, the domain that promotes G<sub>i</sub> binding to MT<sub>1</sub> appears to localize between PDZ9 and PDZ13, since this part of MUPP1 is sufficient to stabilize the ternary complex between MUPP1, G<sub>i</sub>, and MT<sub>1</sub>.

The association of MUPP1 with MT<sub>1</sub> may participate in the high stability of the MT<sub>1</sub>-G<sub>i</sub> protein complex. G<sub>i</sub> has been shown to be precoupled to MT<sub>1</sub> in its inactive form and to remain stably associated upon agonist stimulation despite the presence of high GTP concentrations in intact cells (29, 36). Destabilization of the MT<sub>1</sub>-G<sub>i</sub> complex in the absence of MUPP1 has different effects on the signaling capacities of MT<sub>1</sub>. Whereas MUPP1 is necessary for efficient coupling of MT<sub>1</sub> to the AC pathway, the PTX-sensitive ERK activation is only moderately affected (only when the amount of G<sub>i</sub> proteins becomes limiting). This highlights the potential regulatory role of MUPP1 in the modulation of MT<sub>1</sub> signaling.

MT<sub>1</sub> is not the only GPCR that binds to MUPP1. The serotonin 5-HT<sub>2C</sub> receptor was the first GPCR that has been shown to interact with MUPP1 (41). Although possibly regulated by the phosphorylation state of the receptor, the functional role of 5-HT<sub>2C</sub> binding to PDZ10 of MUPP1 still remains poorly defined (42). Whereas MT<sub>1</sub> and 5-HT<sub>2C</sub> bind to the same PDZ domain (PDZ10), their G protein coupling profiles are different. MT<sub>1</sub> couples preferentially to G<sub>i</sub>, and 5-HT<sub>2C</sub> couples prefer-
the viral protein ORF1 has been shown to promote cytoplasmic sequestration of MUPP1 (23, 48). In both cases, MUPP1 is removed far from its potential membrane partners, thus impeding any interaction with them. Regulation of the MT1/MUPP1 interaction is likely to occur under these circumstances, since functional MT1 expression has been shown in keratinocytes (49) and mammary tumors (50). Recently, MUPP1 has been shown to be robustly up-regulated by hypertonicity and to be important in the osmotic stress response in tight junctions of kidney cells (51). Finally, altered MUPP1 expression levels have been shown in mice with high predisposition to alcohol and barbiturate physical dependence and withdrawal (52). Taken together, MUPP1 expression levels appear to be highly regulated in several physiological and pathological situations.

In conclusion, our study extends the previously panoply of known functions of MUPP1 on GPCR physiology. MUPP1 regulates G protein-dependent GPCR signaling by directly stabilizing the receptor-G protein complex, which may explain the previously reported high stability of the MT1-G complex. Future studies will concentrate on the still largely unexplored functions of the other PDZ domains of MUPP1 on GPCR function.

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