Periplakin Interferes with G Protein Activation by the Melanin-concentrating Hormone Receptor-1 by Binding to the Proximal Segment of the Receptor C-terminal Tail*

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In mice genetic ablation of expression of either melanin-concentrating hormone or the melanin-concentrating hormone-1 receptor results in alterations in energy metabolism and a lean phenotype. There is thus great interest in the function and regulation of this receptor. Using the yeast two-hybrid system we identified an interaction of the actin- and intermediate filament-binding protein periplakin with intracellular C-terminal tail of the melanin-concentrating hormone-1 receptor. Direct association of these proteins was verified in pull-down and coimmunoprecipitation experiments. Truncations and internal deletions delineated the site of interaction to a group of 11 amino acids proximal to transmembrane helix VII, which was distinct from the binding site for the melanin-concentrating hormone-1 receptor-interacting zinc finger protein. Immunohistochemistry demonstrated coexpression of periplakin with melanin-concentrating hormone-1 receptor in specific cells of the pifform cortex, amygdala, and other structures of the adult mouse brain. Coexpression of the melanin-concentrating hormone-1 receptor with periplakin in human embryonic kidney 293 cells did not prevent agonist-mediated internalization of the receptor but did interfere with binding of $^{35}$S-labeled guanosine 5’-3-O-(thio)triphosphate ($^{35}$S)GTP$\gamma$S to the G protein $G_{\alpha\text{11}}$ and the elevation of $[Ca^{2+}]_{i}$. Coexpression of the receptor with the interacting zinc finger protein did not modulate receptor internalization or G protein activation. The interaction of periplakin with receptors was selective. Coexpression of periplakin with the IP prostanoid receptor did not result in coimmunoprecipitation nor interfere with agonist-mediated binding of $^{35}$S)GTP$\gamma$S to the G protein $G_{\alpha}$. Periplakin is the first protein described to modify the capacity of the melanin-concentrating hormone-1 receptor to initiate signal transduction.

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1 The abbreviations used are: MCH, melanin-concentrating hormone; BioTris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxyethyl)propane-1,3-diol; eGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GST, glutathione S-transferase; GTP$\gamma$S, guanosine 5’-3-O-(thio)triphosphate; HA, hemagglutinin; HEK, human embryonic kidney; MAP, mitogen-activated protein; MZIP, MCH-1 receptor interacting zinc finger protein; PBS, phosphate-buffered saline; PPL, periplakin; PPLC, C-terminal 208 amino acid fragment of periplakin, VSV-G, vesicular stomatitis virus glycoprotein; YFP, yellow fluorescent protein.
heterologous cell lines, the MCH-1 receptor is able to regulate signal transduction through both G_{i} and G_{q} coupled pathways (2–6). Despite this, little is known about the regulation of this receptor.

GPCRs generally do not exist in isolation, and there is increasing information on proteins that interact with GPCRs and by so doing alter their cellular distribution or function (22–25). The intracellular C-terminal tail of GPCRs has been particularly well studied in this regard and interacting proteins identified by the application of both proteomic (26, 27) and yeast two-hybrid (28, 29) techniques. We have previously used the MCH-1 receptor C-terminal tail as the bait in yeast two-hybrid screens to identify interactions with a zinc finger protein named MCH-1 receptor-interacting zinc finger protein (MIZIP) (30). Using the same approach we now report the interactions of the MCH-1 receptor with the actin and intermediate filament-binding protein, periplakin (PPL) (31). PPL is a 1,756-amino-acid polypeptide that is expressed widely and at considerable levels in both rodent and human brain (32, 33). Although most actively studied for its role in production of the cornified epithelium in keratinocytes (34–37), we have previously detailed its capacity to interact with splice variants of the human MOP opioid receptor (32). We now map the site of interaction of PPL with the MCH-1 receptor and show that this is distinct from the interactions of the MCH-1 receptor with MIZIP. Interactions between PPL and the MCH-1 receptor are selective but do not modulate MCH-1-mediated internalization of the receptor. This interaction does however, reduce the capacity of the receptor to active G proteins and hence initiate signal transduction. This is the first protein-protein interaction demonstrated to alter agonist-mediated function of the MCH-1 receptor and, as we also demonstrate coexpression of these proteins in specific neurons, is likely to have significance for MCH-mediated functions in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—All materials for tissue culture were supplied by Sigma. [35S]GTP*S (1,250 Ci/mmol) was from PerkinElmer Life Sciences. Other reagents were purchased from Aldrich (Milwaukee, WI) or Boehringer Mannheim (Mannheim, Germany). Mouse monoclonal anti-vesicular stomatitis virus glycoprotein (VSV-G) antibody was from Roche Applied Science. Rabbit polyclonal anti-His antiserum was from Santa Cruz Biotechnology. The sheep anti-GFP antiserum was raised in-house against recombinantly expressed eGFP. The polyclonal sheep anti-PPL antiserum was generated against a 14,000 Da fragment of rMCH-1 receptor (amino acids 299–363) were generated by PCR and cloned into yeast bait vectors. These bait vectors were used to screen a human brain cDNA library in the yeast strain CG1945, screening of a human brain cDNA library in the yeast vector pACTII, and the β-galactosidase filter lift assay were performed as described previously (30).

**Cell Culture and Transient Transfection—**HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) newborn calf serum and 2 mM glutamine. Prior to transfection with plasmid DNA, cells were seeded onto 10-cm² or 6-cm² dishes at 50–70% confluence. Cells were transfected using Lipofectamine reagent (Invitrogen). 48 h later cells were harvested by removal of the growth medium, rinsing twice with ice-cold PBS and scraping in 5 ml of PBS. Cells were pelleted by centrifugation at 2,000 rpm at 4 °C and then stored at –80 °C until membrane preparation.

**Preparation of Membranes—**Cell pellets were resuspended in TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) and ruptured with 50 strokes of a Teflon-on-glass homogenizer. Unbroken cells and nuclei were removed by centrifugation at 1,500 rpm for 5 min. The supernatant fraction was passed through a 25-gauge syringe needle 20 times and then centrifuged for 30 min at 14,000 rpm. These pellets were resuspended in TE buffer and stored at –80 °C until use.

**[35S]GTP*S Binding Assays—**Membranes expressing equivalent levels of VSV-G-hMCH-1 full-length receptor (as determined by densitometric scanning of blots of immunodetected receptors using the anti-VSV-G antibody) were incubated with an assay mix (20 mM HEPES, pH 7.4, 5 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 1.25 mM dithiothreitol, 0.1% bovine serum albumin, 50 mM guanosine 5’-diphosphate, 100 nCi of [35S]GTP*S) containing the indicated concentrations of MCH. Nonspecific binding was determined in the presence of 100 μM GTP*S. Reactions were incubated for 15 min at 30 °C and terminated upon addition of 0.5 ml of stop buffer (20 mM HEPES, pH 7.4, 3 mM MgCl₂, and 100 mM NaCl). The membranes were pelleted by a 15-min centrifugation at 14,000 rpm at 4 °C and resuspended in solubilization buffer (100 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1.25% Nonidet P-40) plus 1% SDS. Samples were prewashed with Pansorbin (Calbiochem) for 1 h at 4 °C and immunoprecipitated overnight at 4 °C with an antiserum against the C-terminal decapetide of Go₅₉ (38) conjugated to protein G-Sepharose. The immunoprecipitates were washed twice with solubilization buffer, and bound [35S]GTP*S was measured by liquid scintillation spectrometry. For equivalent experiments with the IP prostaglandin receptor, membranes were prepared as described above, except that an antiserum (39) that detects the C-terminal decapetide of Go₅₉ was utilized.

**Ca²⁺, and ERK MAP Kinase Assays—**After expression in HEK293 cells of the hMCH-1 receptor in the presence or absence of PPL, the capacity of the MCH receptor to elevate [Ca²⁺], was monitored in single cells as described by Liu et al. (40). Total and phosphorylated/activated forms of ERK2 were detected by Western blotting in such transfected cells were detected by immunoblotting lysates of cells treated for 15 minutes with or without 1 μM MCH with nonselective and phosopho-specific antibodies. Cells were washed in situ twice with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer for 1 h at 4 °C. After centrifugation (15 min, 14,000 × g at 4 °C) to remove cell debris, 10 μg of protein/sample was boiled in 2× Laemmli buffer and resolved by 4–12% BisTris NuPAGE gels and transferred onto nitrocellulose membranes. Total and phosphorylated/activated forms of the ERK1 and ERK2 MAP kinases in these samples were detected by immunoblotting using anti-phospho-ERK and anti-ERK antisera (Cell Signalling) followed by detection with horseradish peroxidase-conjugated secondary antibodies and visualized by ECL (Pierce).

**Co-immunoprecipitation of hMCH-1, MIZIP and PPL-GFP with VSV-G-hMCH-1 Receptor—**24 h post-transfection cells from 6-cm² dishes were split into 6-well plates. The following day the cells were incubated in the presence or absence of agonist (1 μM MCH for VSV-G-hMCH-1 receptor-transfected cells; 1 μM Ioprost for equivalent experiments with the VSV-G-IP prostaglandin receptor) for 30 min. Cells were then washed three times with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, pH 7.5, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 5 mM EDTA, 10 mM NaF, 5% ethylene glycol, and a mixture of protease inhibitors). Insoluble material was removed by a 15-min centrifugation at 14,000 rpm at 4 °C. Equalized amounts of protein were incubated with protein G-Sepharose beads conjugated to mouse monoclonal anti-VSV-G antibody (Roche Applied Science) or sheep polyclonal anti-GFP antiserum for 2 h at 4 °C. The immune complexes were eluted from the beads by the addition of 2×...
Laemmli buffer. Proteins were resolved by SDS-PAGE using 4–12% BisTris NuPAGE gels (Invitrogen) and transferred to nitrocellulose membranes. A rabbit polyclonal anti-His antisemur (Santa Cruz Biotechnology) was used to detect His-MIZIP. VSVG-hMCH-1 receptors were detected using the anti-VSVG-G antibody.

**Receptor Internalization Assay-Biotin Labeling**—HEK293 cells transfected with the cYFP-tagged hMCH-1 receptors were split into 6-well plates and cultured for a further 24 h. Cells were incubated with or without 1 μM MCH for 60 min and then immediately washed twice with ice-cold PBS. The alcohol groups on the cell surface glycoproteins were oxidized to aldehydes by a 30-min incubation at 4 °C with 10 mM sodium m-periodate in PBS containing 1 mM MgCl₂, 0.1 mM CaCl₂. After further washing with PBS, the cells were incubated for 30 min with acetic buffer (0.1 mM sodium acetate, pH 5.5, MgCl₂, 0.1 mM CaCl₂) containing 1 mM Biotin-LC-Hydrazide (Pierce) that reacts with the newly formed aldehyde groups thereby labeling cell surface glycoproteins with biotin. Labeling was terminated by the removal of the biotin solution and washing with PBS. Cells were then solubilized for receptor immunoprecipitation with the sheep polyclonal anti-GFP antisemur as described earlier. Biotin-labeled receptors were detected using horse-radish peroxide-conjugated streptavidin (Pierce) and visualized by ECL.

**Purification of His-Tagged Proteins**—PQE30 plasmids containing the His insertions of PPL or MIZIP were transformed into Escherichia coli BL21 cells. A 10-ml starter culture was used to inoculate 400 ml of LB medium containing 100 μg/ml ampicillin and grown at 37 °C until the culture reached an A₆₀₀ of 0.5. Expression of the His-tagged proteins was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h before cells were harvested by centrifugation at 10,000 rpm for 15 min at 4 °C. Pellets were resuspended in denaturing lysis buffer containing 8 M urea and the His-tagged proteins were purified following the manufacturer’s instructions (Qiagen). Eluted proteins were dialyzed against three changes of PBS containing 5% glycerol at 4 °C over 2 days before storage at –80 °C.

**Purification of GST Fusion Proteins and GST Pull-down Assays**—BL21 cells transformed with the GST fusion constructs were cultured and harvested as described earlier. Pellets were lysed in 20 ml of STE buffer pH 5.0, 150 mM NaCl, 1 mM EDTA containing 0.1 mg/ml lysozyme and incubated with rotation at 4 °C for 1 h. Dithiothreitol was added to a final concentration of 5 mM and 10% sarkosyl in STE buffer to a final concentration of 0.5% was added to lysates before sonication for 1 min. The removal of insoluble material was performed by a 15-min centrifugation at 10,000 rpm at 4 °C. 10% Triton X-100 in STE buffer to a final concentration of 2% and a mixture of protease inhibitors were added to the cleared supernatants before use in GST pull-down assays.

For pull-down assays 0.5–5 ml of lysates was incubated with a 100-μl slurry of 50% (v/v) PBS-washed glutathione-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C. The beads were pelleted by centrifugation (14,000 rpm, 1 min) and washed twice with PBS containing 1% Triton X-100 before the addition of 50 μg of the required His-tagged protein in 1 ml solution of PBS and Triton X-100 containing 5 mM dithiothreitol. After a 2-h incubation at 4 °C, the beads were collected by a 15-min centrifugation at 10,000 rpm at 4 °C. 10% Triton X-100 in STE buffer to a final concentration of 2% and a mixture of protease inhibitors were added to the cleared supernatants before use in GST pull-down assays.

**Confocal Immunofluorescence Staining**—Cells were transfected on coverslips in 6-well plates and the following day were fixed in 4% (v/v) paraformaldehyde in PBS containing 5% sucrose for 10 min at room temperature. Cells were then permeabilized for 10 min in TM buffer (0.15% Triton X-100 and 3% nonfat milk in PBS). Coverslips were incubated for 1 h at room temperature with either 2.5 μg/ml anti-HA antibody (Roche Applied Science) to detect HA-PPL or 2 μg/ml anti-His antibody for His-MIZIP staining. After washing with PBS and TM, coverslips were incubated for 1 h with an Alexa 594-conjugated secondary antibody. After further washing with PBS, coverslips were mounted onto glass slides and observed using a laser-scanning Zeiss LSM510 confocal microscope.

**Fluorescence Immunohistochemistry**—All incubations were performed at room temperature or as indicated. Free floating 50-μm cryostat sections from 4% paraformaldehyde-fixed adult C3H/BL6 mouse brains were permeabilized for 10 min with 0.3% Triton X-100 in PBS, washed with PBS-T (0.05% Triton X-100), postfixed for 30 min with methanol-acetone (1:1) at 4 °C, and incubated in blocking solution (5% horse serum in PBS-T) for 1 h. Sections were incubated for 24 h at 4 °C with blocking solution complemented with an affinity-purified chicken anti-MCH-1 receptor antibody (Chemikon) and the polyclonal sheep anti-PPL antisemur (both diluted 1:1000). After washing with PBS-T, sections were incubated for 2 h with Cy3-conjugated donkey anti-chicken (1:800, Dianova) and Cy3-conjugated donkey anti-sheep (1:400, Sigma). Images were captured with a CCD camera (Hamamatsu) mounted on a Leica Aristoplan fluorescence microscope and analyzed with the Openlab software (Improvision). Brain regions were identified using the mouse brain atlas of Paxinos and Franklin (41) and the rat brain atlas of Paxinos and Watson (42).

**RNA Expression Analysis**—RNA in situ hybridization was performed as described previously (43). In brief, rat postnatal and adult brains were fixed overnight with 4% paraformaldehyde in PBS and sectioned using a cryomicrotome. Radiolabeled antisense and sense RNA probes were generated by in vitro transcription using [α-35S]UTP and linearized cDNAs with nucleotides 1114–1705 of human PPL (cloned from yeast clone L16) and with the full-length open reading frame of rMCH-1 receptor, respectively. Sections were hybridized overnight at 54 °C in a humid chamber, washed with decreasing salt concentrations, dehydrated using increasing ethanol concentrations, and exposed to BetaMax x-ray film (Amersham Biosciences) for 3–7 days.

**RESULTS**

A human brain cDNA library was employed in yeast two-hybrid screens using the sequence corresponding to amino acids 299–353 of the rat MCH-1 receptor as bait. This consists of the 5 amino acids upstream of the highly conserved NPXXY motif of transmembrane region VII and the entire C-terminal tail. From a total of 2.7 × 10⁸ transformed cDNAs, 20 were positive after selection, two of which encoded C-terminal fragments of human PPL (L16, amino acids 1114–1705; L25’, amino acids 1415–1705) (Fig. 1a). The interaction of the C terminus of the rMCH-1 receptor in pAS2 with the yeast clones L16 and L25’ was specific because no growth of yeast was detected using either pAS2 control or the C-terminal regions of hMCH-2 receptor or the rat somatostatin receptor 5 cloned into pAS2 (Fig. 1b). Using deletion mutants of the C terminus of rMCH-1 receptor, we confined the binding of PPL to the prox-
imal C-terminal region of the MCH-1 receptor: growth of yeast on selection medium was not affected by the deletion of the last 28 amino acids of rMCH-1 receptor (MCH-1(299–325)). However, the deletion of the proximal C terminus (MCH-1(319–353)) abolished binding because no growth of yeast on selection medium was detected (Fig. 1b).

To confirm these interactions a GST fusion protein containing amino acids 302–353 of the hMCH-1 receptor was generated and linked to glutathione-Sepharose 4B beads. This was able to capture a His-tagged form of the C-terminal 208 amino acids of PPL (His-PPLC) (Fig. 2a, i). His-PPLC was not captured by glutathione-Sepharose 4B beads to which only GST was linked (Fig. 2a, i). Previous studies (30) have demonstrated an interaction between the C-terminal tail of the rMCH-1 receptor and a zinc finger protein named MIZIP. A His-tagged form of mouse MIZIP was also captured selectively by GST-
hMCH-1 (302–353) (Fig. 2a, ii). In contrast to these results GST fusion proteins incorporating each of hMCH-1 (68–79), hMCH-1 (140–158), and hMCH-1 (233–252), which correspond respectively to the first, second, and third intracellular loops of the receptor (Fig. 2b), all failed to capture His-PPLC or His-MIZIP (Fig. 2a). To address the site(s) of interaction of both His-PPLC and His-MIZIP within the C-terminal region of the hMCH-1 receptor a series of truncations of GST-hMCH-1 (302–353) was produced by sequential removal of 13 amino acid blocks from the C terminus to produce GST-hMCH-1 (302–340), GST-hMCH-1 (302–327), or GST-hMCH-1 (302–314) (b) were expressed and purified. These were used in pull-down assays that also included His-tagged versions of PPLC (a, i) or His-MIZIP (a, ii). Input of the individual GST fusion proteins is shown by protein staining (Ponceau) in the lower panels and capture of the His-tagged proteins in the upper panels. IB, immunoblot.

**Fig. 3.** Interactions of both PPL and MIZIP with the MCH-1 receptor require the proximal region of the receptor C-terminal tail. GST alone or GST fusion proteins incorporating the predicted full-length C-terminal tail (amino acids 302–353) of the hMCH-1 receptor or truncations of this to produce amino acids 302–340, 302–327, or 302–314 (b) were expressed and purified. These were used in pull-down assays that also included His-tagged versions of PPLC (a, i) or His-MIZIP (a, ii). Input of the individual GST fusion proteins is shown by protein staining (Ponceau) in the lower panels and capture of the His-tagged proteins in the upper panels. IB, immunoblot.
(Fig. 3). Both hMCH-1 (302–340) and GST-hMCH-1 (302–327) were able to capture both His-PPLC (Fig. 3a, i) and His-MIZIP (Fig. 3a, ii) as effectively as GST-hMCH-1 (302–353), whereas GST-hMCH-1 (302–314) did not. These studies suggested key interactions to be provided within the sequence 315 CETFRKRLVLSVK 327. GST fusion proteins of hMCH-1 (302–327) were therefore produced which lacked either 316 ETFRK (Δ316–320) or LVLSV (Δ322–326) (Fig. 4). Neither GST-ΔETFRK nor GST-ΔLVLSV was able to capture His-PPLC (Fig. 4a, i), but both retained interactions with His-MIZIP (Fig. 4a, ii), defining distinct sites or modes of binding of these two proteins to the hMCH-1 receptor C-terminal tail.

To extend these studies an N-terminally VSV-G epitope-tagged form of the full-length hMCH-1 receptor was expressed in HEK293 cells along with PPLC tagged at the C terminus with GFP. Immunoprecipitation of PPLC-GFP with an anti-GFP antiserum resulted in coimmunoprecipitation of the 32-kDa VSV-G-hMCH-1 receptor as monitored by immunoblotting such samples with the anti-VSV-G antibody following SDS-PAGE (Fig. 5a). Expression of PPLC-GFP without VSV-G-tagged hMCH-1 receptor did not result in coimmunoprecipitation of this VSV-G-tagged receptor (Fig. 5a). Interaction of PPLC-GFP with the VSV-G-tagged IP

![Figure 4. Elimination of amino acids 316–320 or 322–326 from the C-terminal tail of the MCH-1 receptor prevents interaction with PPL but not with MIZIP.](http://www.jbc.org/Downloaded-from)
prostanoid receptor was not promoted by treatment of the cells with the selective IP prostanoid receptor agonist iloprost (1 μM, 30 min) (Fig. 5a). Similar results were obtained with coexpression of His-MIZIP and the VSV-G-tagged forms of the hMCH-1 and IP prostanoid receptors. Immunoprecipitation of VSV-G-hMCH-1 with the anti-VSV-G antiserum resulted in coimmunoprecipitation of His-MIZIP (Fig. 5b), but coexpression of His-MIZIP with the VSV-G-IP prostanoid receptor followed by immunoprecipitation of the receptor did not result in coimmunoprecipitation of His-MIZIP (Fig. 5b). Again, addition of the IP prostanoid receptor agonist iloprost did not promote interactions between this receptor and His-MIZIP.

The interaction of VSV-G-hMCH-1 receptor with either PPLC-GFP (Fig. 6a) or His-MIZIP (Fig. 6b) was unaffected by treatment with 1 μM MCH for 30 min. In accord with the pull-down experiments, C-terminal truncation of 13 (VSV-G-hMCH-1 340Stop) or 26 (VSV-G-hMCH-1 327Stop) amino acids did not interfere with the coimmunoprecipitation of either PPLC-GFP (Fig. 6a) or His-MIZIP (Fig. 6b), and this was also unaffected by treatment with 1 μM MCH for 30 min.

To explore the functional consequence of interactions of the hMCH-1 receptor with either PPL or MIZIP, the hMCH-1 receptor C-terminally tagged with eYFP was expressed with or without either N-terminally HA-tagged full-length PPL or His-MIZIP in HEK293 cells. Confirmation of successful expression of PPL and MIZIP was obtained by immunoblotting cell lysates (Fig. 7a). Cell surface expression of the hMCH-1 receptor-eYFP was detected in cell surface biotin-
ylation experiments (Fig. 7a). Treatment of the cells with 1 μM MCH for 60 min resulted in internalization of a proportion of the receptor as monitored by the reduction of cell surface receptors available to be biotinylated (Fig. 7a). Coexpression of neither PPL nor MIZIP prevented agonist-mediated receptor internalization (Fig. 7a). By contrast, although an eYFP-tagged form of hMCH-1 receptor 340Stop was internalized in response to the addition of MCH, further deletion to produce eYFP-tagged forms of hMCH-1 327Stop and hMCH-1 314Stop eliminated agonist-mediated internalization (Fig. 7b). Cell surface localization of the eYFP-tagged full-length hMCH-1 receptor and its internalization in response to treatment of the cells with MCH was confirmed further in confocal imaging studies (Fig. 7c).

Key signals from the MCH-1 receptor are mediated via members of the Gi family of G proteins. When coexpressed with a form of the α subunit of Goαi, which is the most highly expressed member of this group of G proteins in the mammalian central nervous system, MCH caused a large, concentration-dependent, enhancement of binding of [35S]GTPγS in Goαi immunoprecipitates which was achieved with EC50 = 350 nM (Fig. 8a). The extent of MCH-mediated activation of Goαi was unaffected by expression of MIZIP (Fig. 8b), but this was reduced substantially in membranes of cells coexpressing the MCH-1 receptor and HA-PPL (Fig. 8b), indicating that PPL was also able to compete with members of the Gq/G11 G protein family to bind to the MCH-1 receptor. By contrast, although MCH produced phosphorylation of the MAP kinases ERK1 and ERK2 in HEK293 cells expressing the MCH-1 receptor, the ability of MCH to cause phosphorylation of these kinases was not compromised by coexpression of either PPL or MIZIP (Fig. 11).

For these experiments to have potential physiological relevance requires the MCH-1 receptor and PPL to be coexpressed in native tissues. Northern blot and reverse transcription-PCR techniques have shown previously that PPL and the MCH-1 receptor are highly expressed within the central nervous system of rodent species (19, 33). To look for overlap of the expression patterns of mRNAs encoding PPL and the MCH-1 receptor in the brain we applied RNA in situ hybridization techniques to analyze the expression of PPL and MCH-1 in adjacent sections of the postnatal rat brain (Fig. 12). At p14, PPL mRNA expression was detected at high levels throughout the brain, especially in the cortex and hippocampus (Fig. 12). In contrast, only low levels of mRNA encoding the MCH-1 receptor were detectable at p14 (Fig. 12). However, in the adult brain, overlap of distribution was detectable in all regions of the brain, especially in the hippocampus and cerebellum (Fig. 12). No signals
were detected when using sense control probes for hybridization (not shown). Such studies, although informative, do not demonstrate coexpression of the MCH-1 receptor and PPL polypeptides in specific cells. We therefore applied fluorescence immunohistochemical techniques to look for colocalization of the MCH-1 receptor and PPL in the adult mouse brain using a sheep polyclonal antiserum raised against the C-terminal 208 amino acids of PPL and an affinity-purified antiserum against the MCH-1 receptor raised in chicken. PPL immunoreactivity was largely restricted to the cytoplasm and plasma membrane of labeled cells (Fig. 13); however, labeling was in addition observed in dendrites, for example, of neurones within the amygdala (arrowheads in Fig. 13d). Colocalization of PPL with the MCH-1 receptor was detected in several regions of the brain, for example, in the cortex, especially at the piriform cortex (Fig. 13, a–c), within the amygdala (Fig. 13, d–f), and the stratum pyramidale of the hippocampus formation (Fig. 13, g–i). In the cerebellum, colocalization of PPL and the MCH-1 receptor was detected in the Purkinje cells, but not in granular cells that express high levels of the MCH-1 receptor but showed only low levels of PPL immunoreactivity (Fig. 13, k–m).

DISCUSSION

It is becoming increasingly clear that GPCRs do not exist in isolation but rather within protein complexes (22–25). Interactions within such complexes can maintain the receptor at a specific location, alter its trafficking properties, or modulate function. The C-terminal tail of many GPCRs encompasses

![Figure 7: MCH-mediated internalization of the MCH-1 receptor is inhibited by C-terminal truncation but not by interactions with PPL or MIZIP.](image-url)
binding sites for interacting proteins, and this has resulted in this region being described as the “magic tail” of GPCRs (44) and “as an anchorage for functional protein networks” (44). A significant number of identified GPCR-interacting proteins also link to the actin cytoskeleton and thus may provide frameworks to define subcellular localization.

PPL is a member of the plakin family of cytolinker proteins (31). Although best studied in keratinocytes, where it plays a key role in providing the basal layer for the construction of the cornified epithelium (36–37), it is both highly expressed and widely distributed in the central nervous system of both human and rodents (32). We recently described the interactions of PPL with the MOP-1 and MOP-1A splice variants of the human MOP opioid receptor (32) and demonstrated this to be a selective interaction because it did not bind to either the β2-adrenoceptor or the α2B-adrenoceptor (32). In the current study we demonstrate selective and high affinity interactions between PPL and the C-terminal tail of a second GPCR, the MCH-1 receptor. The MCH-1 receptor is a well validated target for potential therapeutic intervention in obesity because knock-
out studies in mice have resulted in animals with altered metabolism and a lean phenotype after elimination of expression of either MCH or the MCH-1 receptor (13–15). Treatment of animal models of obesity and type 2 diabetes with either MCH or small molecule antagonists of the MCH-1 receptor also modulate the expression and levels of other key regulators of appetite and energy balance (45, 46). Furthermore, because early antagonists at the MCH-1 receptor have been found to have antidepressant and anxiolytic properties (45, 47) they may find further clinical uses. MCH-1 receptor mRNA and protein are expressed in the ventromedial and dorsomedial nuclei of the hypothalamus, consistent with a role for this GPCR in mediating the effects of MCH on feeding. Equally, because the MCH-1 receptor is expressed in several brain regions (19), in particular those involved in olfactory learning and reinforcement mechanisms, therapies targeting the MCH-1 receptor should act on the neuronal regulation of food consumption.

Identification of PPL as a partner protein for the MCH-1 receptor C-terminal tail in yeast two-hybrid screens was confirmed in both pull-down and coimmunoprecipitation assays. We then defined the region of the receptor responsible as a PPL interacting domain (32). There are, however, a number of differences in this area between the MCH-1 receptor and the MOP opioid receptor. There is a cysteine residue located in the MOP opioid receptor C-terminal tail in a location that in other rhodopsin-like GPCRs generally results in thioacylation (48). At least based on the crystal structure of bovine rhodopsin (49), acylation at this location (50) produces the “fourth intracellular loop” or “helix VIII” of receptors. Data on post-translational thioacylation of the MCH-1 receptor are unclear as to the location of the modification (51), and it should be noted that this cysteine is short section predicted to be proximal to the end of transmembrane helix VII. This region is similar to the section of the MOP opioid receptor identified as a PPL interacting domain (32). We then defined the region of the receptor responsible as a PPL interacting domain (32).
Regulation of the MCH-1 Receptor

reported not to be the target site (51). However, in studies of the interactions between PPL and the MOP opioid receptor we described the interaction as occurring at helix VIII (32). By contrast, the MCH-1 receptor does not have any cysteine residues in the C-terminal tail which are likely sites for thiocacylation. Thus, although this region may adopt a helical structure, such a helix will not be dominated by an acyl chain burying into the membrane bilayer. However, it is noteworthy that both the MCH-1 receptor and the MOP opioid receptor have a proline residue (328 in the hMCH-1 receptor) located immediately at the end of the region we map as the PPL binding site. This helix breaker may define the end of helix VIII in rhodopsin-like GPCRs, which we show not to bind PPL. This helix in helix VIII of many rhodopsin-like GPCRs, including the receptor, although they may overlap to some degree. MIZIP was unable to interfere with either agonist-mediated internalization of the full-length MCH-1 receptor or G protein activation. By contrast, coexpression of PPL with the MCH-1 receptor resulted in a substantial loss of G protein activation. The MCH-1 receptor is known to interact with and activate members of the G and G protein subfamilies and by monitoring both agonist-mediated loading of [GSS iGTP]S onto the α subunit of Gαi and the elevation of intracellular [Ca2+] we demonstrate that the presence of PPL is able to limit activation of both G protein classes. As noted earlier, a rational explanation for this can be suggested based on the known contribution of the proximal section of C-terminal tail, i.e. helix VIII, in G protein interaction and activation (53). Mutation of the two basic residues within the ETFRK sequence located between 316 and 320 of the MCH-1 receptor has recently been reported to interfere with agonist-mediated calcium elevation (54), and we now show both that elimination of the ETFRK sequence prevents interaction with PPL and that coexpression of PPL with the hMCH-1 receptor interferes with MCH-mediated elevation of intracellular [Ca2+] . It is thus likely that PPL competes with both families of G proteins for a common binding site on this receptor. In contrast to these results, after expression of the hMCH-1 in HEK293 cells, MCH-mediated phosphorylation of the MAP kinases ERK1 and ERK2 was not abrogated by coexpression of PPL. Virtually all GPCRs are able to promote phosphorylation and activation of these kinases, and this can occur via a wide range of mechanisms, some of which appear not to require G protein activation (55, 56). Moreover, ERK1 and ERK2 activation in HEK293 cells is a highly sensitive and amplified response in which only a very small fraction of the total G-G protein pool requires activation to generate a maximal signal (for example, see Ref. 57). Thus, if hMCH-1 receptor stimulation of ERK phosphorylation is a G protein-mediated effect in these cells, expression of PPL would have to be extremely high to compete sufficiently to modulate or block this response.

Overlapping distributions of mRNAs encoding the MCH-1 receptor and PPL were observed in rat brain, and, more directly, coexpression of the two proteins was also demonstrated in neurons in a range of brain regions and structures, suggesting likely interactions of these proteins in vivo. These interactions may therefore modify the physiological actions of MCH and the MCH-1 receptor.

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Periplakin Interferes with G Protein Activation by the Melanin-concentrating Hormone Receptor-1 by Binding to the Proximal Segment of the Receptor C-terminal Tail

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