The Basic Residues in the Membrane-Proximal C-Terminal Tail of the Rat Melanin-Concentrating Hormone Receptor 1 Are Required for Receptor Function

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Melanin-concentrating hormone (MCH) is a hypothalamic neuropeptide that plays a key role in food intake. It acts through two G protein-coupled receptors (GPCRs), MCH1R and MCH2R, of which MCH1R is the primary regulator of food intake. We have previously reported that N-linked glycosylation of the extracellular domain of MCH1R is necessary for cell surface expression and signal transduction. We now report a role for the rat MCH1R C-terminal region. We constructed serial C-terminal truncation mutants and determined the resulting changes in protein expression, cell surface expression, ligand binding, and MCH-stimulated calcium influx. By analyzing two mutants, ∆T317 (deletion of 36 C-terminal amino acids) and ∆R321 (deletion of 32 C-terminal amino acids), we found that the region between Phe318 and Arg321 was responsible for signal transduction. A more detailed analysis was performed with single or multiple residue mutations. Single mutations of Arg319, Lys320, or Arg321 exhibited a decrease in the cell surface expression, whereas mutations of either Arg319 or Lys320, but not Arg321, showed a significant reduction in the calcium influx. Furthermore, simultaneous mutations of Arg319 and Lys320 produced a pronounced decrease in the efficacy of calcium influx stimulation compared with single mutations. A computational analysis revealed a dibasic amino acid motif that is conserved among many class 1 GPCRs and may be part of the amphiphilic cytoplasmic helix 8 (an eight-cytoplasmic helix). Our results therefore provide new insights into the role of the putative helix 8 in the regulation of GPCR function. (Endocrinology 145: 3712–3723, 2004)

MELANIN-CONCENTRATING HORMONE (MCH) is a 19-amino acid cyclic peptide that was first isolated from the pituitary gland of salmon (1). It has subsequently been detected in rats (2), where it is predominantly expressed in the cell bodies of the lateral hypothalamus and is widely distributed throughout the central nervous system (3). In mammals, MCH plays a major role in the regulation of feeding behavior and energy balance. The levels of MCH mRNA increase with fasting and in genetically obese ob/ob mice (4). Intracerebroventricular administration of MCH stimulates feeding behavior in rats (4). Importantly, targeted disruption of the MCH gene in mice results in a lean phenotype due to hypophagia and an increased metabolic rate (5). In contrast, transgenic mice overexpressing MCH show obesity and resistance to insulin (6).

The orphan G protein-coupled receptor (GPCR), somatostatin-like receptor 1 (7), is activated by MCH and belongs to the class 1 GPCRs (8–12). This receptor, now referred to as MCH1R, is highly expressed in the brain (13, 14). MCH elevates intracellular Ca2+ levels, inhibits forskolin-stimulated cAMP production, and activates MAPK in cells transfected with MCH1R (9, 10, 11, 15). MCH can also activate a similar receptor, MCH2R (16). However, several nonhuman species, including rodents, have no functional MCH2R or encode a nonfunctional MCH2R pseudogene (17). Because MCH1R-deficient mice are lean, hyperactive, and hyperphagic and have an altered metabolism (18, 19), MCH1R is viewed, at least in rodents, as the physiologically relevant MCH receptor for energy homeostasis. In support of this, recent studies have shown that selective MCH1R antagonists inhibit MCH-induced food consumption in rats (20, 21), although one of these antagonists also exhibited antidepressant and anxiolytic effects (21). These results suggest that the MCH-MCH1R system has a crucial role in feeding behavior, and that inhibition of this system is an attractive pharmacological target for the treatment of obesity and some mental disorders. Despite the potential therapeutic importance of MCH1R, our understanding of the mechanisms underlying the activation of MCH1R is limited. Biochemical analyses have identified two amino acid residues important for ligand binding: Asp123 in the third transmembrane domain (22), and Asn23 in the extracellular amino-terminal region (23). N-Linked glycosylation of Asn23 is necessary for both expres-

Abbreviations: Bmax, Maximum binding; EC50, 50% effective concentration; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; helix 8, eight-cytoplasmic helix; HMMER, hidden Markov model software package; MCH, melanin-concentrating hormone; MCH1R, melanin-concentrating hormone receptor 1; MCH2R, melanin-concentrating hormone receptor 2; Pfam, protein family hand-curated HMMER model; PLCβ, phospholipase Cβ; PTX, pertussis toxin; 7TM, seventh transmembrane domain.

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sion on the cell surface and ligand binding (23). However, nothing is known about the structural motifs or amino acids necessary for signal transduction or agonist-induced internalization of MCH1R.

It has previously been shown that the cytoplasmic C-terminal region of GPCRs plays an important role in receptor trafficking (24–27), intracellular signaling (27–29), dimerization (30), and agonist-induced receptor internalization (31). Several reports have shown that amino acids in the intracellular loop and C-terminus are cooperatively involved in G protein coupling (32, 33). Mutagenesis studies have attempted to identify the amino acids of different GPCRs that are involved in receptor activity. Some of the C-terminal tail amino acid residues involved in effector coupling and cell surface expression have been identified in several receptors. A couple of conserved motifs in the C terminus have been proposed, but the C-terminal tail is variable in length, and the definitive sequences involved in trafficking and/or signal transduction remain unclear. For example, the basic residues and the cysteine cluster in the C-terminal tail function cooperatively to optimize surface expression in CCR5 (24), whereas disoleucine or the FxxxFxxx sequence in the proximal C-terminal tail is responsible for the cell surface expression of the melanocortin-4 receptor (25) or the dopamine D1 receptor (26), respectively.

Different lines of evidence have suggested the importance of the proximal C-terminal domain adjacent to the seventh transmembrane domain (7TM). The first atomic resolution structural analysis of bovine rhodopsin defined the existence of a short amphipathic helix, named the eight-cytoplasmic helix (helix 8). This helix runs parallel to the cytoplasmic surface of the cell membrane and has a carboxyl terminus that is probably formed by the insertion of two palmitoyl groups in two Cys residues into the membrane bilayer (34). Studies using site-directed mutants in combination with biochemical and biophysical assays showed that the N terminus of the helix 8 in rhodopsin is part of the binding site for the C terminus of Gαs and plays a role in the regulation of the βγ-subunits (35, 36). Furthermore, a more recent study with a leukotriene receptor revealed that helix 8 is responsible for the conformational change after G protein activation (37).

In view of the potential therapeutic importance of MCH1R, we examined the structural elements in the C-terminal domain of MCH1R that are required for protein expression, cell surface expression, radioligand binding, and MCH-induced calcium influx. For this purpose we constructed a series of C-terminal-truncated mutants and also analyzed mutants with substituted groups of amino acids in the proximal C-terminal tail. We provide evidence that basic residues located in the membrane-proximal C-terminal region after the 7TM are required for cell surface expression and signaling in the rat MCH1R. We also discuss the conserved properties of dibasic residues in the proximal C-terminus among many class 1 GPCRs.

**Materials and Methods**

cDNA constructs for the MCH1R with mutagenesis

Incorporation of a sequence encoding the Flag epitope tag before the first methionine in rat MCH1R was performed by PCR (23). The purified full-length cDNA of MCH1R was subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). All C-terminal truncated mutants, ΔT317, AR321, ΔQ325, ΔQ333, and ΔT342, were produced by replacing the codon of the original amino acid with a stop codon using the wild-type receptor cDNA as a template (Fig. 1A). Oligonucleotide-mediated, site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The substitution sites are shown in Fig. 1B. A total of four separate sites were targeted for glutamine or alanine substitution. Residues Phe318, Arg319, Lys320, and Arg321 were chosen for our studies, and each residue in the full-length MCH1R was mutated to glutamine, glutamic acid, or alanine. To elucidate the cumulative effects, three double mutants, R319Q/K320Q, R319Q/R321Q, and K320Q/R321Q, and a triple mutant, R319Q/K320Q/R321Q, were constructed. Mutations in the MCH1R cDNA sequence were confirmed by sequencing analysis. The mutated MCH1R cDNA was excised with EcoRI and XhoI enzymes and inserted into the expression vector pcDNA3.1.

**Cell culture and transfection**

DNA was mixed with Lipofectamine Plus transfection reagents (Invitrogen), and the mixture was diluted with OptiMEM and added to 70–80% confluent human embryonic kidney cells (HEK293T) plated on six-well plates. The transfected cells were then cultured in DMEM containing 10% fetal bovine serum (FBS). Forty-eight hours after the transfection, cell membranes were prepared from the cells for radioligand binding assays. For the calcium influx assay, FACScan flow cytometric analysis and immunocytochemistry, the cells were placed onto 96-well plates, 24-well plates, and coverslips, respectively, 24 h after the transfection and then cultured for another 24 h at 37 °C. Flag-MCH1R and R319Q/K320Q/R321Q were also stably transfected into HEK293T cells. After 72 h, transfected cells were selected in the presence of zeocin at a final concentration of 0.4 μg/ml for 3 wk and used for measurement of the cAMP level or for the internalization assay. We were unable to obtain stable expressions of ΔT317, AR321, and ΔS325 in HEK293T cells.

**Western blotting and immunoprecipitation**

To generate whole cell extracts, HEK293T cells were lysed with an ice-cold sodium dodecyl sulfate sample buffer (50 mm Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 50 mm β-mercaptoethanol, and 10% glycerol), and the lysates were then homogenized by sonication (Bioruptor-UCD-320TM, Toshiba Ltd., Yokohama, Japan) using four 30-sec bursts at 25% full power at 4 °C. To detect the level of glycosylation more clearly, an immunoprecipitation analysis was performed (23). HEK293T cells were lysed with a rabbit polyclonal in ice-cold solution A (50 mm Tris-HCl (pH 8.0), 150 mm NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, and a protease inhibitor mixture (Roche, Indianapolis, IN)) for 20 min at 4 °C, and the lysates were then cleared by centrifugation at 18,500 × g for 20 min at 4 °C. For immunoprecipitation, aliquots of cell lysates (50 μg protein) were preclarified with 30 μl protein G-agarose (50% suspension in PBS) on a rotator at 4 °C for 30 min. The protein G-agarose was then removed by centrifuging the lysates at 18,500 × g for 5 min at 4 °C. Subsequently, the preclarified cell lysates were incubated with 2 μg anti-Flag M2 antibody (Sigma-Aldrich Corp., St. Louis, MO) and protein G-agarose on a rotator at 15 h at 4 °C. The immunecomplexes were washed three times with solution A once with PBS, and subsequently eluted from the protein G-agarose by the addition of 30 μl sodium dodecyl sulfate sample buffer. Proteins were separated in a 12.5% SDS-PAGE gel and electrophoretically transferred to a Hybond-P polyvinylidene difluoride membrane (Amersham International, Little Chalfont, UK). After blocking with 5% skim milk dissolved in washing buffer (0.2% Tween 20 in Tris-HCl-buffered saline), Flag-MCH1R on the membrane was detected using the anti-Flag M2 antibody (Sigma-Aldrich Corp., St. Louis, MO) and protein G-agarose on a rotator at 15 h at 4 °C.

**FACScan flow cytometric analysis of cell surface receptors**

Transfected HEK293T cells in 24-well plates were fixed with 1% paraformaldehyde for 10 min at room temperature, then incubated with 8 μg/ml anti-Flag M2 antibody in PBS containing 20% FBS for 1 h. The cells were washed three times with PBS and then incubated with 10
fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG secondary antibody for 1 h. The cells were collected from the wells with 5 mM EDTA and analyzed using a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA). Cells were gated by light scatter or exclusion of propidium iodide, and 10,000 cells were acquired for each time point. The mean fluorescence of all cells minus the mean cell fluorescence with the FITC-conjugated secondary antibody only was used for the calculations.

Confocal immunofluorescence microscopy

Transfected HEK293T cells were fixed in ice-cold PBS and centrifuged at 1000 x g for 5 min. The cell pellet was homogenized in ice-cold 50 mM Tris-Cl buffer (pH 7.4) containing 5 mM EDTA and ultracentrifuged twice at 48,000 x g for 20 min each time at 4 C. The pellets were
then suspended in 50 mm Tris-HCl (pH 7.4) buffer containing 5 mm EDTA and used as the membrane fractions. The membrane fractions (30 μg protein for each assay) were incubated with increasing concentrations of [125I]Phel17,18-MCH (Amer sham International) from 0.01-5 mm in the absence or presence of 1 μm nonlabeled MCH (Peptide Institute, Osaka, Japan) in 500 μl assay buffer [50 mm Tris-HCl (pH 7.4), 1 μm phosphoramidon, 0.5 mm phenylmethylsulfonyl fluoride, and 0.2% BSA] at room temperature for 2 h. The binding reaction was terminated by rapid filtration through GF/C glass filter plates (Whatman International, Ltd., Maidstone, UK) presoaked in 0.2% polyethylenimine, followed by three washes with 3 ml PBS. The radioactivity retained in the filter was determined using a γ-counter. Specific binding was defined as the difference between total binding and nonspecific binding.

Measurement of intracellular Ca²⁺

Transfected HEK293T cells seeded on black-walled, 96-well plates (BD Biosciences, Franklin Lakes, NJ) were loaded for 1 h at 37°C with a nonwash calcium dye (Calcium Assay Kit, Molecular Devices, Sunnyvale, CA) in Hanks' balanced salt solution containing 20 mm HEPES (pH 7.5). For each concentration of MCH, the level of intracellular Ca²⁺ was determined by rapid filtration through GF/C glass filter plates (Whatman International, Ltd., Maidstone, UK) presoaked in 0.2% polyethylenimine, followed by three washes with 3 ml PBS. The radioactivity retained in the filter was determined using a γ-counter. Specific binding was defined as the difference between total binding and nonspecific binding.

Measurement of cAMP production

Transfected HEK293T cells were seeded on 24-well plates and incubated for 24 h. The cells were preincubated with cAMP assay buffer (Hanks' balanced salt solution supplemented with 20 mm HEPES and 0.3 mm 3-isobutyl-1-methyxanthine, pH 7.5) for 10 min. The cells were then incubated with forskolin (1 μm) and various concentrations of MCH for 15 min. Reactions were terminated with 0.3 n HCl, and the level of intracellular cAMP was measured using a RIA kit (Yamasa, Kyoto, Japan) following the manufacturer's protocol.

Sequence searches

For sequence searches and identification of the end of the 7TM domain in GPCRs, we used protein family hand-curated HMMER model (Pfam, protein family databases for alignment and HMMs, PF00001 rhodopsin family) as the database (http://sanger.ac.uk/Software/Pfam/index.shtml) and HMMER (profile HMMs for protein sequence analysis) as the analysis tool (http://hmmer.wustl.edu/). Pfam is a large collection of protein multiple sequence alignments and profile hidden Markov models (profile HMMs). Profile HMMs can be used to perform sensitive database searching using statistical descriptions of a family's consensus sequence.

**Results**

**The C-terminal tail of MCH1R is necessary for its surface expression and ligand binding**

To analyze the function of the C terminus, a series of C-terminal truncated receptors were generated, as shown schematically in Fig. 1A. Amino-terminal Flag-tagged rat MCH1R (Flag-MCH1R) was transfected into HEK293T cells to measure the level of protein expression, surface expression and localization of the receptor proteins. Wild-type MCH1R and Flag-MCH1R have similar EC₅₀ values for MCH (23). The C-terminal tail of the rat MCH1R extends for 42 residues from the plasma membrane (residues 312-353) and was progressively truncated to generate five truncated mutants, ΔT317, AR321, ΔS325, ΔQ333, and ΔT342, in which the number represents the last residue. These mutants were obtained by PCR as described in Materials and Methods. The levels of receptor expression were determined from immunoblotting analysis of the transfected cells using the anti-Flag M2 antibody (Fig. 2A). Several immunoreactive bands were detected in the whole cell lysates of cells transfected with wild-type Flag-MCH1R. The results of our previous enzymatic deglycosylation study suggest that the smaller band beneath the 38.5-kDa band is the nonglycosylated form, whereas the other higher molecular mass bands represent different N-linked glycosylated forms of MCH1R (23). Immunoreactive bands were also detected in the cells transfected with the five C-terminal truncated mutants. A progressive reduction in the apparent molecular weights of the two major bands between 38.5 and 47.6 kDa in Flag-MCH1R followed the C-terminal truncations (Fig. 2A). The intensities of the bands

**Fig. 2. Expression of Flag-MCH1R and mutant receptors in HEK293T cells.** A, Transfected cells were lysed with sodium dodecyl sulfate sample buffer, and 30 μg total protein were separated by 15% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with an anti-Flag M2 antibody. Several major immunoreactive bands are detected in Flag-MCH1R. B, Immunoprecipitation of cell lysates from cells transfected with C-terminus-truncated mutants using the anti-Flag M2 antibody. Cell lysates were generated by centrifugation as described in Materials and Methods. Several major immunoreactive bands were detected in Flag-MCH1R, whereas the intensity of the higher molecular mass band (arrowhead) was decreased in the truncated mutants. C, Immunoprecipitation of cell lysates from cells transfected substituted mutants in the proximal C-terminal tail using the anti-Flag M2 antibody. The arrowhead indicates the location of an immunoreactive band that shows a reduction in intensity in the mutant receptors.
corresponding to these two bands in the truncated mutants were quantified by imaging analysis in three different immunoblotting experiments, and the expression levels of the bands in the five C-terminal-truncated mutants were comparable to those of Flag-MCH1R. For most of the truncated mutants, however, the expression pattern in the immunoblotting differed somewhat from that of Flag-MCH1R. The smaller band beneath 38.5 kDa showed a relatively higher intensity in the presence of 1 h in the absence or presence of unlabeled MCH in an assay buffer. Nonspecific binding was quantified in the presence of 1 μM unlabeled MCH, and specific binding was defined as the difference between total and nonspecific binding.

The data represent the mean ± SEM of four independent experiments performed in duplicate.

$^a P < 0.05$ comparing ΔS325 and ΔQ333, by t test.

### TABLE 2. Specific radioligand binding of Flag-MCH1R and mutant receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flag-MCH1R</td>
<td>1.6 ± 0.2</td>
<td>568.0 ± 110.6</td>
</tr>
<tr>
<td>ΔT317</td>
<td>2.0 ± 0.1</td>
<td>232.9 ± 27.0</td>
</tr>
<tr>
<td>ΔR321</td>
<td>2.5 ± 0.3</td>
<td>227.0 ± 28.3</td>
</tr>
<tr>
<td>ΔS325</td>
<td>1.7 ± 0.2</td>
<td>240.1 ± 23.0</td>
</tr>
<tr>
<td>ΔQ333</td>
<td>1.7 ± 0.1</td>
<td>300.8 ± 11.5</td>
</tr>
<tr>
<td>ΔT342</td>
<td>2.2 ± 0.3</td>
<td>352.5 ± 43.2</td>
</tr>
</tbody>
</table>

The membrane fractions were incubated with increasing concentrations of [125I]Phe13, Tyr19-MCH in the absence or presence of unlabeled MCH in an assay buffer. Nonspecific binding was quantified in the absence of 1 μM unlabeled MCH, and specific binding was defined as the difference between total and nonspecific binding.

$^a P < 0.05$ comparing ΔS325 and ΔQ333, by t test.

ΔT317, ΔR321, and ΔQ333. Indeed, ΔT342 surface fluorescence was only slightly decreased compared with that of Flag-MCH1R. The relative intensity of membrane targeting decreased in the following order: Flag-MCH1R > ΔQ333 > ΔR321 = ΔT317. In cells permeabilized with Triton X-100, all of the mutants displayed a similar level of intracellular reactivity, but their labeling patterns were distinctly different. Both ΔT342 and Flag-MCH1R were localized to the plasma membrane, whereas ΔT317, ΔR321, and ΔQ333 were distributed in the perinuclear zone and cytoplasm. Both the expression level and the distribution of ΔS325 were similar to those of ΔR321 (data not shown). These results suggest that the first half of the residues in the C-terminal tail (by Q333) is not sufficient for trafficking of receptors to the plasma membrane.

**Importance of the proximal region of MCH1R for effector coupling**

We next assessed whether the C-terminal domain of MCH1R plays a role in stimulating the influx of calcium. HEK293T cells transiently transfected with Flag-MCH1R or truncated mutants were stimulated by MCH, and the resultant calcium influx was quantified using a Flexstation. A representative curve is illustrated in Fig. 4. Cells expressing the shortest mutant, ΔT317, did not express functional receptors, whereas MCH-mediated calcium influx was present in cells expressing the other truncated mutants (ΔR321, ΔS325, Q333, and ΔT342). The fact that ΔT317 did not respond to MCH is noteworthy, because we have shown that...
the expression patterns in immunoblotting, surface expression, and ligand binding of ΔT317 were almost equivalent to those of ΔR321. Table 1 shows the EC_{50} values for Flag-MCH1R and the truncated mutants. ΔR321 and ΔS325 exhibited a maximal response that was reduced by 47–48% and had 5-fold higher EC_{50} values than Flag-MCH1R. ΔQ333 had a nearly identical EC_{50} value to MCH, but its maximal response was reduced by 33%. ΔT342 had a 40% lower EC_{50} value, and the maximal response was reduced by 21%. These results show that the maximal response in the calcium influx

**Fig. 3.** Confocal immunolocalization of Flag-MCH1R and the mutant receptors with an anti-Flag M2 antibody. The cell surface expressions were compared using transfected nonpermeabilized cells (−TX, without Triton X-100; left) and permeabilized cells (+TX, with Triton X-100; right). ΔS325 possessed similar features to ΔR321 in both nonpermeabilized and permeabilized cells (data not shown). Vector-transfected cells incubated with the anti-Flag M2 antibody showed no significant staining (data not shown). Bar, 10 μm.
The decreases in intensity were comparable to those observed in R319Q/K320Q and R319Q/K320Q/R321Q (data not shown). The lower level of expression in the higher molecular mass band seems to be due to a lack of appropriate glycosylation of the receptor, as described for the case of the C-terminal truncated mutants.

The cell surface expressions of the 14 mutants were measured by flow cytometric analysis (Table 3). In F318A, in which the phenylalanine residue at position 318 was replaced with an alanine residue, the surface expression was essentially identical to that of Flag-MCH1R. Replacing single amino acids at 319–321 with glutamine decreased the levels of cell surface expression by 30–40% compared with that of Flag-MCH1R. Another series of single-substituted mutants with glutamic acid also showed 20–25% decreases in their cell surface expressions. These results show that replacement of proximal basic residues with a negatively charged amino acid, glutamic acid, does not generate a more pronounced reduction in cell surface expression compared with replacement with glutamine. The three double mutants and one triple mutant exhibited marked reductions in cell surface expression. The cell surface expressions of R319Q/K320Q, R319Q/R321Q, K320Q/R321Q, and R319Q/K320Q/R321Q were reduced by 55%, 42%, 50%, and 56%, respectively. The expression levels of R319Q/K320Q and R319Q/K320Q/R321Q at the cell surface were nearly identical to that of ΔT317.

The mutants R319Q/K320Q and R319Q/K320Q/R321Q displayed high affinity saturable binding of [125I]Phe13,Tyr19-MCH, with Kᵢ values of 1.2 ± 0.4 and 1.5 ± 0.3 nM (mean ± sem from three independent experiments), respectively. These values are comparable to that obtained for Flag-MCH1R (Table 2). The Bmax values for R319Q/K320Q and R319Q/K320Q/R321Q were reduced by approximately 60%. These values roughly resembled those found for ΔT317 and ΔR321. Confocal microscopy revealed that these mutants were localized to the perinuclear zone and cytoplasm rather than the plasma membrane, and that the intracellular region was identical to ΔT317 (data not shown). Because positive charges appear to directly coordinate surface expression, we

Identification of residues contributing to the signaling pathway of the receptor for MCH

To further elucidate the role of the proximal 318–321 region, single or multiple residues within this domain were targeted for mutagenesis, as shown in Fig. 1B. Immunoblotting analysis of whole cell extracts of 14 mutants showed several immunoreactive bands, and the expression patterns were relatively similar to that of Flag-MCH1R. The higher accumulation of the smaller band beneath 38.5 kDa that was observed in ΔT317, ΔR321, and ΔS325 was not detected in any of the substituted receptors. However, the intensity of the upper band above 47.6 kDa was decreased in 10 mutants, as assessed by immunoprecipitation analysis. This band exhibited a reduction in single-substituted mutants in which the positively charged basic amino acids (Arg319, Lys320, and Arg321) were replaced with the noncharged amino acid residue, glutamine. Imaging analysis revealed that the intensity of the corresponding band was reduced by 31%, 35%, and 31% in R319Q, K320Q, and R321Q, respectively (Fig. 1C). In R319E, K320E, and R321E (replacement to the negatively charged glutamic acid), this band exhibited less than 20% reduction in intensity (data not shown). Profound reductions of the corresponding band were evident in the three double mutants and one triple mutant substituted at 319–321. The intensity of the band in R319Q/K320Q and R319Q/K320Q/R321Q was decreased by 62% and 72%, respectively (Fig. 2C). The decreases in intensity were comparable to those observed in ΔT317, ΔR321, and ΔS325 (Fig. 2B). Other double mutants (R319Q/R321Q and K320Q/R321Q) showed expression patterns similar to that of R319Q/K320Q (data not shown). Approximately corresponded to the cell surface expression level assessed by FACScan flow cytometry, and that ΔT317 was the only mutant to lose its ability to induce signal transduction. This implies that the proximal C-terminal region between Phe318 and Arg321 is crucial for signal transduction in the MCH-activated calcium influx.

**TABLE 3.** Sequence requirements for cell surface expression and calcium signaling of various mutants of the proximal C-terminal tail of MCH1R

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell surface expression (FACS; %)</th>
<th>Maximum response (%)</th>
<th>EC₅₀ of MCH (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flag-MCH1R</td>
<td>100</td>
<td>100</td>
<td>5.5 ± 1.8</td>
</tr>
<tr>
<td>R318A</td>
<td>94.6 ± 4.8</td>
<td>93.8 ± 9.4</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>R319Q</td>
<td>64.5 ± 2.4</td>
<td>81.6 ± 2.9</td>
<td>19.8 ± 3.4</td>
</tr>
<tr>
<td>R320Q</td>
<td>63.5 ± 9.6</td>
<td>83.4 ± 8.8</td>
<td>14.5 ± 2.0</td>
</tr>
<tr>
<td>R321Q</td>
<td>70.0 ± 7.7</td>
<td>80.7 ± 1.8</td>
<td>7.4 ± 0.6</td>
</tr>
<tr>
<td>R319E</td>
<td>75.9 ± 14.4</td>
<td>70.6 ± 3.1</td>
<td>39.4 ± 2.4</td>
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<tr>
<td>R320E</td>
<td>80.6 ± 9.3</td>
<td>81.3 ± 3.0</td>
<td>23.0 ± 3.3</td>
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<tr>
<td>R321E</td>
<td>78.1 ± 7.8</td>
<td>80.9 ± 4.8</td>
<td>8.8 ± 0.8</td>
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<td>R319Q/K320Q</td>
<td>45.8 ± 0.5</td>
<td>50.6 ± 3.1</td>
<td>50.1 ± 14.4</td>
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<tr>
<td>R319Q/R321Q</td>
<td>60.5 ± 5.4</td>
<td>79.4 ± 4.8</td>
<td>37.4 ± 7.6</td>
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<td>K320Q/R321Q</td>
<td>58.2 ± 4.9</td>
<td>80.1 ± 2.6</td>
<td>26.1 ± 3.3</td>
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<tr>
<td>R319Q/K320Q/R321Q</td>
<td>44.2 ± 3.7</td>
<td>41.3 ± 9.8</td>
<td>264.4 ± 38.4</td>
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<td>R319K/R321K</td>
<td>100.5 ± 24.7</td>
<td>90.7 ± 8.5</td>
<td>6.6 ± 0.6</td>
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<tr>
<td>F318R</td>
<td>ND</td>
<td>107.3 ± 5.1</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>R321 + R</td>
<td>ND</td>
<td>108.3 ± 5.9</td>
<td>8.1 ± 4.1</td>
</tr>
</tbody>
</table>

The data represent the mean ± SEM of four independent experiments, performed in duplicate.

ND, Not determined.
also replaced the amino acids at positions 319 and 321 with lysine. However, no significant change in the surface expression was detected between Flag-MCH1R and R319K/R321K.

We next investigated the effects of these mutants on the signal transduction pathway coupled to the calcium influx (Table 3). In F318A, the EC_{50} value for the calcium influx was significantly reduced by 66% compared with that of Flag-MCH1R. Both R319Q and K320Q had EC_{50} values that were approximately 3-fold greater than that of Flag-MCH1R. In R321Q, the maximal calcium influx was reduced by 20%, although the mutant displayed a similar EC_{50} value as Flag-MCH1R. The critical importance of positions 319 and 320 in the calcium influx was suggested by the results with single-substituted mutants with glutamic acid. R319E and K320E showed 7- and 4-fold higher EC_{50} values, respectively, whereas R321E showed no significantly impaired function. The double mutants, R319Q/K320Q, R319Q/R321Q, and K320Q/R321Q, had 9-, 7-, and 5-fold higher EC_{50} values, respectively (Table 3). These data suggest that the impairment of the calcium influx is dependent on G_{i/o} activity. Next, we characterized the phenotype of R319Q/K320Q/R321Q by measuring the cAMP level after application of MCH to stably transfected cells (Fig. 5). MCH potently stimulated the cAMP level in Flag-MCH1R transfected cells. In contrast, cells that expressed R319Q/K320Q/R321Q had an 18-fold higher EC_{50} value (30.2 nM) compared with the maximal response. The EC_{50} value in PTX-treated cells was 11.2 ± 0.9 nM, whereas that in untreated cells was 4.9 ± 0.6 nM (data are the mean ± SEM of three independent experiments), and the +PTX (G_{i/o}-dependent response)/−PTX (G_{i/o}-independent response) ratio was 2.30. These results indicate that both PTX-sensitive G_{i/o} and PTX-insensitive G_{i/o} mediate the mobilization of intracellular calcium in response to MCH1, and that a large component of the calcium influx is dependent on G_{i/o} activity rather than G_{i/o} activity. Furthermore, we characterized the phenotype of R319Q/K320Q/R321Q by measuring the cAMP level after application of MCH to stably transfected cells (Fig. 5). MCH potently stimulated forskolin-stimulated cAMP accumulation in Flag-MCH1R with an EC_{50} value of 1.6 ± 0.5 nM in Flag-MCH1R transfected cells. In contrast, cells that expressed R319Q/K320Q/R321Q had an 18-fold higher EC_{50} value (30.2 ± 2.6 nM). The maximal inhibition of cAMP accumulation with 1 μM MCH was

![Figure 5](https://academic.oup.com/endo/article-abstract/145/8/3712/2878135/3719)
60% in Flag-MCH1R-transfected cells, whereas it was 15% in cells transfected with R319Q/K320Q/R321Q. The basal level and increase in the cAMP level induced by forskolin in R319Q/K320Q/R321Q-transfected cells were similar to those induced in Flag-MCH1R-transfected cells (data not shown). It can be concluded therefore that R319Q/K320Q/R321Q affects the coupling of both calcium signaling and adenylyl cyclase.

Discussion

Mutagenesis studies have demonstrated the importance of the cytoplasmic C-terminal region of GPCRs for a variety of receptor functions, including cell surface expression, signaling pathways, dimerization, agonist-induced receptor internalization, and desensitization. Recent studies have addressed the role of the proximal C-terminal tail in receptor function (27–28, 35–37, 39). A dihydrophobic motif or a C-terminal FxxxFxmx motif, which is relatively conserved in the proximal C terminus, has been found to be involved in the normal surface expression of the melanocortin-4 receptor (27) or the endoplasmic reticulum export signal for the dopamine D1 receptor (28), respectively. In rhodopsin, three amino acids (Asn, Lys, and Gln) in the N terminus of helix 8 have been identified to interact directly with the C terminus of Go protein (35, 36). In view of the therapeutic importance of the MCH1R receptor, we undertook a study to determine whether its C-tail function and amino acids are responsible for receptor function. Five deletion mutants and 14 substitution mutants were synthesized and used to identify amino acid residues essential for receptor function. Our data demonstrate crucial roles for the basic residues Arg319, Lys320, and Arg321 present in the proximal C-terminal region of GPCRs.

It has previously been shown that the efficacy of a ligand in signal transduction is partially dependent on the density of the target receptors on the cell surface (40). To determine the receptor expression of the C-terminal-truncated mutants on the cells, Western blotting, FACS analysis, and immunofluorescence studies were performed. Immunoprecipitation experiments revealed a reduction in the intensity of only the higher molecular mass band for ΔT317, ΔR321, ΔS325, and ΔQ333 compared with Flag-MCH1R (Fig. 2B), and this may be due to the altered glycosylation (23). The expression levels on the cell surface varied among the mutants (Table 1). The cell surface expressions of the mutants ΔT342 and ΔQ333 were reduced by 27% and 42%, respectively, compared with wild-type Flag-MCH1R. In contrast, the expressions of ΔT317, ΔR321, and ΔS325 were reduced by nearly 54%. Further, analysis by radioligand binding showed that these three mutants exhibited very similar binding parameters (Kd and Bmax in Table 2). However, we found that ΔT317 lost the capacity to stimulate a calcium influx, whereas ΔR321 remained functional. The abolished coupling of ΔT317 was not due to either the extent of glycosylation or the cell surface expression level, because these were identical to the values for ΔR321. Instead, these results indicate that residues 318–321 in the membrane-proximal domain are critical for signaling. Our present finding implies the functional importance of the membrane-proximal domain in the C terminus for signal transduction and cell surface expression, in accordance with other studies (25–27, 35–37). Next, we identified the residues in the membrane-proximal domain that have critical roles in signaling in MCH1R.

First, we showed that addition of the four amino acids (Phe318Arg319Lys320Arg321) to the C terminus of ΔT317 resulted in recovery of the signaling capability, suggesting that G proteins require these amino acids for coupling. Then, we constructed mutants with substitutions at positions 318–321. All three of the basic residues, Arg319, Lys320, and Arg321, appeared to play equivalent roles in cell surface expression, because the expression levels in the single-substituted mutants were significantly reduced to similar levels. In contrast, Arg319 and Lys320 were found to be potential sites of signal transduction in the single-substituted mutants. Replacement of Arg319 and Lys320 with glutamine or glutamic acid showed significantly higher EC50 values than that of Flag-MCH, whereas two mutants substituted at Arg321 exhibited no significance differences. R319Q/K320Q and R319Q/K320Q/R321Q showed impaired signal function. Transfection of lower amounts of DNA into the cells resulted in lower levels of cell surface expression, but the EC50 values in the calcium influx remained similar. Furthermore, the magnitudes of the reductions in cell surface expression and glycosylation in R319Q/K320Q and R319Q/K320Q/R321Q were nearly identical to those in ΔR321 and ΔS325. Thus, it is unlikely that the drastic decreases in signal function observed in R319Q/K320Q and R319Q/K320Q/R321Q are primarily caused by the altered glycosylation or low level of cell surface expression. On the other hand, the precise role of Phe318 in MCH1R is currently unknown. Because substitution of Phe318 in MCH1R resulted in a modest influence on signaling, even though the efficacy was significantly increased in the mutants, it can be speculated that Phe318 may play a minor tuning role in MCH1R signal transduction in the calcium influx.

Several different structural determinants within transmembrane or cytoplasmic domains have been shown to influence proper G protein recognition, yet there is no definitive rule that defines receptor-G protein interactions. For example, both the hydrophobic and basic residues in the second and third cytoplasmic loops of α7-nicotinic receptors may be involved in Gαq coupling (41), whereas muscarinic receptors and α2-adrenergic receptors require basic residues in the third intracellular loop for selective activation of Gαi (42, 43). In contrast, the C-terminal tail of prostaglandin EP3 receptors determines the G protein specificity (28). Although Flag-MCH1R is able to couple with both Gq and Gi/o, the R319Q/K320Q/R321Q mutant showed a nearly identical +PTX/−PTX ratio to that of Flag-MCH1R in the calcium influx. If proximal basic amino acid residues are related to the G protein coupling specificity, the +PTX/−PTX ratio in R319Q/K320Q/R321Q should be dramatically changed compared with that in Flag-MCH1R. Furthermore, the R319Q/K320Q/R321Q mutant showed markedly impaired coupling to both the calcium influx and adenylyl cyclase inhibition. This suggests that the basic region in the proximal C-terminal tail of MCH1R is not involved in the G protein coupling specificity. Additional studies will be necessary to identify the amino acid residues required for the selective coupling of MCH1R to Gαq or Gαi/o proteins.
Our experiments implicate another region of the C-terminal tail in playing a supportive role in receptor function. The triple mutant, R319Q/K320Q/R321Q, showed markedly impaired function in the MCH-activated calcium influx, but its phenotype was different from that of /H9004 T317, in which receptor coupling was completely lost. This suggests that a C-terminal domain different from the proximal basic region may have an additional role in receptor function. Immuno-precipitation experiments showed lower amounts of glycosylation of the higher molecular mass band in /H9004 R321 and /H9004 S325 than in /H9004 Q333 (Fig. 2B). Next, we found significant differences in the cell surface expression levels and Bmax values between ΔS325 and ΔQ333. These results may account for previous reports that glycosylation of the receptor is required for proper folding, receptor trafficking, and eventually cell surface expression (23, 38). In terms of the receptor-mediated calcium influx caused by MCH, the EC50 values for ΔR321 and ΔS325 were 5-fold higher that that of Flag-MCH1R, whereas that of ΔQ333 was almost identical. Thus, these results suggest that the domain between positions 326 and 333 plays a role in efficient targeting to the cell surface and ultimately in its ability to activate calcium influx with the appropriate sensitivity to MCH. Moreover, we have preliminary data showing that internalization is impaired in /H9004 Q333 even though this mutant had nearly the same EC50 value for calcium influx as authentic Flag-MCH1R (Saito, Y., unpublished observation). This implies that there may be little correlation between MCH1R-mediated activation of the sig-

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**Fig. 6.** Stacked histograms of the amino acid position numbers of the basic amino acids in the C termini of mouse and human class I GPCRs. Two hundred and one mouse (A) and 223 human (B) class I GPCRs were scanned for the occurrence of all amino acid residues within the first 30 residues of the C-terminal tail, and the occurrence of basic residues (His, Lys, and Arg). As shown in both figures, the position of the basic residues peaks at 5, 8–9, and 12–13 residues from the end of the 7TM domain, and dibasic residues most frequently occur at positions 8–9. For sequence searches and identification of the end of the 7TM domains in GPCRs, we used Pfam (PF00001 rhodopsin family) as the database (http://sanger.ac.uk/Software/Pfam/index.shtml) and HMMER (profile HMMs for protein sequence analysis) as the analysis tool (http://hmmer.wustl.edu/).
naling pathway and receptor internalization. This conclusion is consistent with the data reported for the pituitary adenylate cyclase activating polypeptide type 1 receptor and indicates that the proximal C-terminus mediates signal transduction whereas the distal region is involved in internalization (29).

The mutants in which the total net charge was modified, R319K/R321K, F318R, and R321K, could still be expressed at the cell surface and were able to couple. These observations imply that a moderate level of positive charge in the proximal C-terminus region in MCH1R is sufficient for surface expression and signaling. One question that arises from our data is how the basic amino acid residues in the membrane-proximal domain contribute to the receptor function. A possible explanation for this might involve the existence of a helix 8 in MCH1R, consistent with the fact that MCH1R belongs to the rhodopsin subfamily class 1 GPCRs. Based on the structure of bovine rhodopsin (34), the Psipred2 program predicts that the proximal C terminus of MCH1R forms an amphipathic helix from E316 to S325, whereas the discrimination of protein secondary structure class method predicts the structure of bovine rhodopsin (34), the Psipred2 program. Both models predict that the basic residues R319–R321 would be present in the middle region of helix 8 in MCH1R. In helix 8 of rhodopsin, the charged/polar groups are clustered on one side, whereas the hydrophobic groups are on the other side (34). Mutations of three amino acids (Asn-Lys-Clin) at the N terminus of this loop led to a dramatic decrease in the ability of rhodopsin to activate Gα, and further affected the regulation of βγ-subunit binding (35, 36). Interestingly, preventing palmitoylation of two Cys residues in helix 8 did not affect to appear as Gα interaction (35). Thus, a charged/polar amino group is the helix propagation site of helix 8 and plays a pivotal role in its structure-function relationship in rhodopsin (44, 45). By analogy, it can be speculated that the basic clusters in helix 8 of MCH1R may be necessary to constitute a proper structure of helix 8 that potentially represents a site with electrostatic charge and shape complementary to G proteins.

Finally, to estimate the general implication of the basic residues in the proximal C terminus of MCH1R, we surveyed the amino acid position numbers of the basic amino acids in the C terminus of 201 mouse class 1 GPCRs and 223 human class 1 GPCRs within the first 30 residues (Fig. 6). This analysis revealed specific features of the C-terminal tail in the first 15 residues from the end of the 7TM in mouse and human GPCRs; positively charged residues are located at positions 5, 8–9, and 12–13 in the C-terminal tail, and the most frequent starting point is at positions 8–9, the second dibasic motif. Mouse and human rhodopsins have a conserved Lys that occurs at position 5 in the C terminus, whereas rat, mouse, and human MCH1Rs have a conserved dibasic motif, Arg, Lys, that corresponds to positions 8 and 9. The complete conservation between mouse and humans may indicate the general functional importance of the basic residues in the proximal C-terminal tail in GPCRs.

In conclusion, this report provides the first evidence that the basic residues in the membrane-proximal C-terminal tail, which are conserved among many class 1 GPCRs, are involved in MCH1R function. The present study extends our knowledge of the molecular determinants of the MCH1R-G protein interface. Furthermore, the conserved characteristics in the C terminus, as shown in Fig. 6, provide an initial basis from which future investigations can determine the molecular interactions underlying other GPCRs. Future characterization of receptor mutants together with structural analyses will ultimately be necessary to define the complex receptor-G protein interaction.

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References

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35. Krishna AG, Menon ST, Tarry TJ, Sakmar TP 2002 Evidence that helix 8 of rhodopsin acts as a membrane-dependent conformational switch. Biochemistry 41:8298–8309


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