Molecular Determinants of Tuberooinfundibular Peptide of 39 Residues (TIP39) Selectivity for the Parathyroid Hormone-2 (PTH2) Receptor

N-Terminal Truncation of Tip39 Reverses PTH2 Receptor/PTH1 Receptor Binding Selectivity*†

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Tuberoinfundibular peptide of 39 residues (TIP39) and the parathyroid hormone-2 (PTH2) receptor form part of an extended family of related signaling molecules that includes the PTH1 receptor, which responds to PTH and PTH-related protein. TIP39 does not appreciably activate the PTH1 receptor, but in this study it is shown to bind the receptor with moderate affinity (59 nM). In this study, we investigated the molecular determinants of both ligand and receptor for the PTH2 receptor selectivity of TIP39 and quantitatively evaluated the role of molecular elements in the binding of TIP39 to the PTH2 and PTH1 receptors. A chimeric receptor composed of the N-terminal extracellular domain of the PTH1 receptor and the remainder (juxtamembrane domain) of the PTH2 receptor (P2-NP1) was fully activated by TIP39 (E₉⁰ ≈ 98% of the rPTH-(1–34), E₉⁰, EC₅₀ = 2.0 nM). This receptor chimera bound TIP39 with an equivalent affinity to the wild-type PTH2 receptor (2.3 and 2.0 nM, respectively). The reciprocal chimeric receptor (P1-NP2) was not activated by TIP39 and bound the ligand with an affinity equivalent to that of the PTH1 receptor. Thus, the juxtamembrane receptor domain specifies the signaling and binding selectivity of TIP39 for the PTH2 receptor over the PTH1 receptor. Removing six N-terminal residues of TIP39 eliminated activation of the PTH2 receptor and reduced binding affinity 70-fold. In contrast, this truncation increased affinity for the PTH1 receptor 10-fold, reversing the PTH2/PTH1 receptor binding selectivity and resulting in a high affinity interaction of TIP-(7–39) with the PTH1 receptor (6 nM). These findings can be explained by a strong interaction between the N-terminal region of TIP39 and the juxtamembrane domain of the PTH2 receptor, with the corresponding domain of the PTH1 receptor acting as a selectivity barrier against high affinity binding of TIP39. As a result, TIP-(7–39) is a highly potent, selective antagonist for the PTH1 receptor.

Tuberooinfundibular peptide of 39 residues (TIP39) is a recently discovered neuropeptide that was purified from bovine hypothalamus on the basis of its ability to activate the PTH2 receptor (1). TIP39 is a good candidate for the PTH2 receptor’s endogenous ligand. It strongly activates the human, rat, and zebrafish² PTH2 receptors (1). PTH also strongly activates the human PTH2 receptor (2), but it is only a weak partial agonist for the rat (3) and zebrafish² receptors. The physiological roles of TIP39 and the PTH2 receptor are currently being investigated. The PTH2 receptor is most abundant in the nervous system. Its expression is relatively high in the hypothalamus, where nerve terminals in the median eminence and cell bodies in the periventricular nucleus have particularly high receptor levels, suggesting a role in the modulation of pituitary function (1). PTH2 receptor concentration in the superficial lamina of the spinal cord dorsal horn suggests a role in the modulation of pain perception (1). In the periphery, the receptor is expressed by discrete cells in a number of tissues including pancreatic islet somatostatin cells, heart and vascular muscle cells, and cells within bronchioles and vasculature in the lung (4).

The PTH2 receptor and TIP39 form a part of an extended family of related receptors and ligands (1). The human PTH2 receptor shares 51% amino acid sequence identity with the human PTH1 receptor. The PTH1 receptor mediates the principal actions of PTH (elevation of blood calcium levels) and PTHrP (a locally acting autocrine/paracrine factor and developmental regulator) (5, 6). Both PTH receptors belong to the type II family of G-protein-coupled receptors that respond to peptide modulators, including calcitonin, glucagon, secretin, and vasoactive intestinal polypeptide. The similarity identified for PTH receptors extends to their ligands (Fig. 1). Five residues are identical when the sequences of TIP39, PTH, and PTHrP are aligned. TIP39 is somewhat more similar to PTH. Seven of the 19 C-terminal amino acids are identical between bovine TIP39 and PTH from most species. The PTH2 and PTH1 receptors, together with their ligands, have presumably evolved to selectively mediate different physiological functions. In this regard, the PTH1 receptor mediates the responses to PTH and PTHrP (6) but does not respond to TIP39 (1), whereas the PTH2 receptor responds to TIP39 and perhaps PTH but not to PTHrP (1).

The molecular basis of PTHrP selectivity for the PTH1 receptor over the PTH2 receptor has been studied extensively (7–11). For the PTH1 receptor, these and other selectivity studies (12–16) have been used to propose models of the molecular determinants of PTH1 receptor selectivity. For PTH2 receptors, there are few potential selectivity determinants. TIP39 is somewhat more similar to PTH. It is somewhat more similar to PTH2 receptors than PTHrP and PTH does not activate the PTH2 receptors (1). TIP39 is a good candidate for the PTH2 receptor’s endogenous ligand. It strongly activates the human, rat, and zebrafish² PTH2 receptors (1). PTH also strongly activates the human PTH2 receptor (2), but it is only a weak partial agonist for the rat (3) and zebrafish² receptors. The physiological roles of TIP39 and the PTH2 receptor are currently being investigated.

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FIG. 1. Amino acid sequence alignment of bovine TIP39 with the N-terminal sequence of bPTH and human PTHrP (hPTHrP). Residues common to all three sequences are boxed. Residues common to only bTIP39 and bPTH are enclosed by the dashed box, and additional residues common to only bPTH and PTHrP are underlined.

The receptor interactions of TIP39 have not previously been examined, beyond the initial observation of selective activation of the PTH2 receptor. We have now begun investigating the molecular basis of TIP39 selectivity for the PTH2 receptor over the PTH1 receptor. Previous studies of PTH receptors and other type II G-protein-coupled receptors (19–22). Receptor-ligand cross-linking studies have confirmed this binding orientation for ligand binding to the PTH1 receptor (17, 23, 24) and also for PTH binding to the human PTH2 receptor (25).

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EXPERIMENTAL PROCEDURES

Reagents and Peptides—The following peptides were purchased from Bachem (Torrance, CA) or Peninsula Laboratories (Belmont, CA): rPTH(1–34), [Nle8,21,Tyr34]rPTH(1–34) amid, [Nle8,18,Tyr34]bPTH(3–34) amid, bTIP39 was obtained from AnaSpec Inc. (San Jose, CA) or Biomolecules Midwest (Waterloo, IL). The letters “r” and “b” designate the peptide sequence as rat and bovine, respectively. The peptides were dissolved in 10 mM acetic acid, with the concentration calculated using the peptide content elements in the binding of TIP39 to the PTH2 and PTH1 receptors. For preparation of transfected COS-7 cell membranes, confluent 15-cm tissue culture plates were transfected with 30–100 μg of DNA, and cells were harvested 3 days after transfection. For preparation of transfected COS-7 cell membranes, confluent 15-cm tissue culture plates were transfected with 30–100 μg of DNA, and cells were harvested 3 days after transfection.

Measurement of Ligand-stimulated cAMP Accumulation—Ligand-stimulated accumulation of cAMP was measured as described previously (3), using a radioimmunoassay to quantify cAMP (9).

Isolation of Cell Membranes—P2 membrane preparations from HEK293 cells expressing the human PTH2 or PTH1 receptors were isolated as described previously (30). COS-7 cell membranes were prepared using a radioimmunoassay to quantify cAMP. In all these assays, a very low concentration of radioligand was measured by displacement of radioligand binding. Three methods were employed. An assay employing centrifugation to separate bound and free radioligand was used to accurately measure ligand binding parameters (30). A higher throughput method employing rapid filtration was used to generate comparative ligand binding data (30). Whole-cell binding assays (9) were used to measure radioligand binding to chimeric PTH2/glucagon receptors. In each of these assays, the IC50 closely approximates the ligand affinity. In the centrifugation assay, cell membranes (45–50 μg), radioligand (100,000–300,000 cpn), and unlabeled ligand were incubated in a final volume of 1 ml of assay buffer (10 mM HEPES, 100 mM NaCl, 1 mM

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EDTA, 3 mM MgSO₄, pH 7.5, supplemented with 0.3% nonfat dried milk powder, 100 μM (4(2-aminoethyl) benzene sulfonamide fluoride, and 1 μg/ml bacitracin) for 2 h at 21 °C. Membranes were collected at 18,000 × g, the surface of the pellet was gently washed, and the radioactivity was counted as described previously (30). For the PTH1 receptor, [125I]-[Nle8,18,Tyr34]bPTH-(3–34) was used as radioligand at a radioactivity was counted as described previously (30). For the PTH1 receptor, [125I]-[Nle8,21,Tyr34]rPTH-(1–34)) and unlabeled ligand were incubated for these experiments. Greater than 50% of the total radioligand was bound if all of the membrane in the incubation was bound within the membrane pellet. For 125I-TIP39 binding to the PTH2 receptor in HEK293 membranes, this requirement necessitated the use of 15 μg of membrane protein from transfected cells, made up to 45 μg with membranes from nontransfected HEK293 cells. (Greater than 50% of the total radioligand was bound if all of the membrane in the incubation was from transfected cells).

In the filtration assay 5–10 μg of membrane protein, 50,000–100,000 cpm of radioligand (56–112 pm for 125I-TIP39 and 28–56 pm for 125I-[Nle8,21,Tyr34]rPTH-(1–34)), and unlabeled ligand were incubated for 2 h at 21 °C. Membranes were harvested as described (30). Total binding was less than 15% of the total amount of radioactivity added. The whole-cell binding assay was performed as described previously (9).

Data Analysis—Concentration dependence data for ligand-stimulated cAMP accumulation and displacement of radioligand binding were analyzed using the following four-parameter logistic equation using Prism 2.01 (GraphPad Software Inc., San Diego, CA).

\[ y = \min + (\max - \min)/(1 + 10^{(X - Xc)/d}) \]  

where X represents the logarithm of the ligand concentration and \( \min \) represents the pseudo-Hill slope. For cAMP accumulation, \( y \) represents the amount of cAMP produced at a given peptide concentration, \( \max \) is the cAMP level in the absence of ligand, and \( \max \) is the maximum level produced. For inhibition of radioligand binding, \( y \) is the cpm bound at a given unlabeled radioligand concentration, \( \max \) is nonspecific binding (measured in the presence of a high concentration of the unlabeled version of the radiolabeled ligand), and \( \max \) is total binding (measured in the absence of unlabeled ligand). Statistical comparison of multiple means was performed initially by single factor analysis of variance followed by post hoc analysis with the Newman-Keuls test. Statistical comparison of two means was performed using a two-tailed Student’s t test.

RESULTS

Binding of TIP39 and rPTH-(1–34) to PTH2 and PTH1 Receptors—In HEK293 cells, the stably expressed human PTH2 receptor (293PTH2 receptor) is potently activated by TIP39 (EC₅₀ = 0.44 nM) and by rPTH-(1–34) (EC₅₀ = 58 pm, \( \max = 85\% \) of the response to TIP39), whereas PTHrP-(1–34) is much less potent (Fig. 2A). The human PTH1 receptor stably expressed in HEK293 cells (293PTH1 receptor) is potently activated by rPTH-(1–34) and PTHrP-(1–34) (EC₅₀ values of 0.55 and 0.44 nM, respectively) but not by TIP39 (Fig. 3A). TIP39 therefore selectively activates the PTH2 receptor in HEK293 cells. This activation profile closely resembles that of the receptors transiently expressed in COS-7 cells (1). It is possible that TIP39 binds to the PTH1 receptor but fails to activate it. It is also not clear how closely related are the concentration dependences of TIP39 activation and binding. We therefore measured the binding of TIP39 to PTH1 and PTH2 receptors. The binding assays were performed in the absence and presence of 10 μM GTPγS to determine whether ligand binding was sensitive to receptor-G-protein (R-G) coupling.

125I-[Nle8,21,Tyr34]rPTH-(1–34) (125I-rPTH-(1–34)) has been used previously as a radioligand for the PTH2 receptor (9, 10), but we found that the signal-to-noise ratio was low in membrane binding assays (typically 3.5- and 2-fold in the absence and presence of GTPγS, respectively). Since TIP39 is a potent agonist for the PTH2 receptor, we evaluated it as a radioligand. TIP39 contains a tyrosine residue at position 29 that can be radiiodinated as well as a methionine residue at position 30 that can potentially be oxidized during iodination. TIP39 labeled in a chloramine-T catalyzed reaction did not bind detectably to the PTH2 receptor. 125I-TIP39 prepared in a lactose peroxidase-catalyzed reaction bound to the PTH2 receptor in HEK293 membranes with a considerably higher signal-to-noise ratio than 125I-rPTH-(1–34) (20- and 15-fold in the absence and presence of GTPγS, respectively), and no specific binding was detected in membranes prepared from nontransfected HEK293 cells. Unlabeled TIP39 displaced 125I-TIP39 binding to the PTH2 receptor with high potency (IC₅₀ = 0.59 nM, Fig. 2B, Table I). The presence of 10 μM GTPγS produced a parallel 4.7-fold rightward shift of the binding curve, suggesting that TIP39 binds with higher affinity to the R-G complex than to the uncoupled receptor (Fig. 2B). The pseudo-Hill slope for TIP39 was significantly less than unity (Table I). The increase of the slope was observed by rebinding or probing the 2-h incubation time (data not shown). At the PTH1 receptor, TIP39 completely inhibited 125I-[Nle8,18,Tyr34bPTH-(3–34) binding with a moderate affinity of 59 nM, the binding curve described by a pseudo-Hill slope of unity (Fig. 3B, Table I). Binding was insensitive to GTPγS, indicating that the ligand binds with indistinguishable affinity to the R-G and R states of the receptor (Fig. 3B, Table I). TIP39 therefore binds selectively to the PTH2 receptor over the PTH1 receptor. The pep-
Table I

Binding of TIP39 and rPTH-(1–34) to PTH2 and PTH1 receptors in HEK293 membranes

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Radioligand</th>
<th>10 μM GTPyS present</th>
<th>bTIP39</th>
<th>rPTH-(1–34)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>−logIC₅₀ (IC₅₀)</td>
<td>Pseudo-Hill slope</td>
</tr>
<tr>
<td>PTH2</td>
<td>125I-bTIP39</td>
<td>No</td>
<td>9.23 ± 0.13</td>
<td>0.60 ± 0.04</td>
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<tr>
<td>PTH2</td>
<td>125I-bTIP39</td>
<td>Yes</td>
<td>8.56 ± 0.12</td>
<td>0.59 ± 0.03</td>
</tr>
<tr>
<td>PTH2</td>
<td>125I-PTH-(1–34)</td>
<td>No</td>
<td>9.09 ± 0.17</td>
<td>0.58 ± 0.05</td>
</tr>
<tr>
<td>PTH1</td>
<td>125I-bPTH-(3–34)</td>
<td>No</td>
<td>7.23 ± 0.08</td>
<td>1.16 ± 0.16</td>
</tr>
<tr>
<td>PTH1</td>
<td>125I-bPTH-(3–34)</td>
<td>Yes</td>
<td>7.23 ± 0.08</td>
<td>1.24 ± 0.20</td>
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</table>

Activation of Chimeric PTH2/PTH1 receptors by TIP39 and rPTH-(1–34)—Chimeric PTH2/PTH1 receptors were used to examine the molecular determinants of the receptor that specify the PTH2 receptor signaling selectivity of TIP39. These receptors were constructed by exchanging between the human PTH2 and PTH1 receptors a region comprising transmembrane domains 2–7 and the intervening loops (including the first intracellular loop) and the C-terminal tail, collectively referred to as the juxtamembrane domain. Chimeric and wild-type receptors were expressed in COS-7 cells, and ligand-stimulated cAMP accumulation was measured.

The four receptors studied (chimeric and wild type) produced an equivalent maximal accumulation of cAMP in response to rPTH-(1–34) and were activated with an equivalent potency (EC₅₀) by this ligand (Fig. 4, Table II). As described previously (1), the PTH2 receptor was fully and potently activated by TIP39 in COS-7 cells, whereas the ligand produced no detectable response at the PTH1 receptor (Fig. 4, Table II). A chimeric receptor made up of the juxtamembrane region of the PTH2 receptor and N-terminal extracellular domain of the PTH1 receptor (P2-NP1) was also fully activated by TIP39 (Fig. 4C, Table II); the maximal cAMP accumulation was 98% of that for rPTH-(1–34) at the same receptor. TIP39 activated this receptor with high potency (EC₅₀ = 2.0 nM), slightly lower than the potency of this ligand at the wild-type PTH2 receptor (EC₅₀ = 0.42 nM). The reciprocal chimera P1-NP2 containing the juxtamembrane domain of the PTH1 receptor was not detectably activated by TIP39 (Fig. 4D). These findings indicate that the juxtamembrane receptor region specifies the PTH2/PTH1 receptor signaling selectivity of TIP39.

Binding of TIP39 and rPTH-(1–34) to Chimeric PTH2/PTH1 Receptors—We examined the molecular determinants of the receptor specifying TIP39's binding selectivity for the PTH2 receptor using the chimeric PTH2/PTH1 receptors described above. rPTH-(1–34) bound with high affinity to wild-type and chimeric PTH receptors in COS-7 membranes (Fig. 5, Table III), suggesting that the conformation of the chimeric receptors was not greatly disrupted compared with that of the wild-type receptors. The P2-NP1 chimera, comprised of the juxtamembrane domain of the PTH2 receptor and N-terminal region of the PTH1 receptor, bound TIP39 with high potency (IC₅₀ = 2.3 nM) that was not significantly different from the TIP39 IC₅₀ at the wild-type PTH2 receptor (2.0 nM) (Fig. 5, Table III). The reciprocal chimeric receptor (P1-NP2, containing the juxtamembrane domain of the PTH1 receptor) bound TIP39 with low affinity (IC₅₀ = 280 nM), comparable with the ligand's affinity for the wild-type PTH1 receptor (160 nM) (Fig. 5, Table III). Therefore, the juxtamembrane domain of the PTH2 receptor specifies the PTH2 receptor binding selectivity of TIP39 as well as specifying the signaling selectivity (Fig. 4, Table II). For the PTH2 and P2-NP1 receptors, the pseudo-Hill slope value was less than 1 whereas the value for the PTH1 and P1-NP2 receptors was approximately unity. TIP39 completely inhibited binding of 125I-rPTH-(1–34) to the PTH1 and P2-NP2 receptors, and rPTH-(1–34) completely displaced 125I-TIP39 binding to the PTH2 and P2-NP1 receptors (Table III).

The receptor states identified in these binding assays were evaluated using GTPyS to promote receptor/G-protein dissociation (31). GTPyS (10 μM) reduced radioligand binding by 53 ± 4%, 5 ± 2%, 69 ± 6%, and 63 ± 1% at the PTH2, PTH1, P2-NP1, and P1-NP2 receptors, respectively. Thus, for the chimeric receptors and the PTH2 receptor, the predominant state identified in these assays was the receptor-G-protein complex.
FIG. 4. Activation of chimeric PTH2/PTH1 receptors and wild-type PTH receptors by TIP39 and [Nle<sup>8,21</sup>,Tyr<sup>34</sup>]rPTH-(1–34). Wild-type and chimeric receptors were expressed in COS-7 cells. Ligand-stimulated cAMP was measured as described under “Experimental Procedures” (●, TIP39; ○, [Nle<sup>8,21</sup>,Tyr<sup>34</sup>]rPTH-(1–34)). A, PTH2 receptor; B, PTH1 receptor. C, chimeric receptor composed of N-terminal domain and first transmembrane domain of the PTH1 receptor fused to the remainder of the PTH2 receptor (P2-NP1). D, chimeric receptor composed of the N-terminal domain and first transmembrane domain of the PTH2 receptor fused to the remainder of the PTH1 receptor (P1-NP2).

TABLE II
Parameters of rPTH-(1–34) and TIP39 stimulation of cAMP production in COS-7 cells transiently expressing chimeric PTH2/PTH1 receptors and wild-type PTH receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>rPTH-(1–34)</th>
<th>TIP39</th>
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<tr>
<td></td>
<td>logEC&lt;sub&gt;50&lt;/sub&gt; (EC&lt;sub&gt;50&lt;/sub&gt;)</td>
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<tr>
<td></td>
<td>nM pmol · well&lt;sup&gt;−1&lt;/sup&gt;</td>
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<tr>
<td>P₁</td>
<td>9.62 ± 0.17&lt;sup&gt;a&lt;/sup&gt; 3.9 ± 0.4&lt;sup&gt;b&lt;/sup&gt; (0.24)</td>
<td>&gt;300 2.6 ± 1.3&lt;sup&gt;±&lt;/sup&gt;</td>
</tr>
<tr>
<td>P₂-NP₁</td>
<td>9.58 ± 0.15&lt;sup&gt;a&lt;/sup&gt; 3.9 ± 0.3&lt;sup&gt;b&lt;/sup&gt; (0.26)</td>
<td>&gt;300 0.1 ± 1.0&lt;sup&gt;±&lt;/sup&gt;</td>
</tr>
<tr>
<td>P₂</td>
<td>9.69 ± 0.13&lt;sup&gt;a&lt;/sup&gt; 4.7 ± 1.2&lt;sup&gt;b&lt;/sup&gt; (0.20)</td>
<td>9.38 ± 0.10&lt;sup&gt;c&lt;/sup&gt; (0.42) 92 ± 6</td>
</tr>
<tr>
<td>P₂-NP₁</td>
<td>9.49 ± 0.12&lt;sup&gt;a&lt;/sup&gt; 4.8 ± 1.2&lt;sup&gt;b&lt;/sup&gt; (0.32)</td>
<td>8.70 ± 0.16&lt;sup&gt;c&lt;/sup&gt; (2.0) 98 ± 10</td>
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</table>

The state of the PTH1 receptor identified in the assay cannot be defined unambiguously. However, the R-G coupling status of this receptor is not relevant to the evaluation of ligand binding selectivity given that TIP39 does not detectably discriminate the R-G complex from the uncoupled receptor (Fig. 3B, Table I). These considerations suggest that the juxtamembrane domain specifies TIP39’s PTH2/PTH1 receptor binding selectivity under conditions in which the receptor-G-protein complex is the receptor state predominantly detected in the binding assay. The signal/noise ratio did not permit critical evaluation of the binding selectivity of the chimeric receptors in the presence of GTPγS. However, in whole cell binding assays, in which the receptor is probably predominantly uncoupled from G-protein (26), the juxtamembrane domain again specifies the PTH2/PTH1 receptor binding selectivity of TIP39 (IC<sub>50</sub> values for the PTH2, PTH1, P2-NP1, and P1-NP2 receptors of 3.3, 415, 5.4, and 1600 nM, respectively; graphical data not shown).

Effect of N-terminal Truncation of TIP39 on Stimulation of cAMP Production and Ligand Binding at PTH2 and PTH1 Receptors—The above evaluation of chimeric PTH2/PTH1 receptors indicated that the juxtamembrane region of the receptor specified both the signaling selectivity and binding selectivity of TIP39 for the PTH2 receptor over the PTH1 receptor. In studies of the orientation of ligand binding to type II G-protein-coupled receptors, the N-terminal region of the ligand has been shown to interact with the juxtamembrane domain of the receptor, leading to receptor activation and second messenger generation (8, 12, 13, 21). We therefore tested whether the N-terminal region of TIP39 was required for receptor activation by measuring the effects of removing residues from its N-terminus on ligand-stimulated adenyl cyclase activity. We also measured the extent to which the N-terminal region of TIP39 specifies the selective binding of the ligand to the PTH2 receptor by measuring the binding affinity of N-terminally truncated ligands for the PTH2 and PTH1 receptors.

At the 293PTH2 receptor, deletion of one, two, or four residues from the N terminus of TIP39 reduced the potency for stimulation of cAMP accumulation but did not affect the maximal ligand-stimulated adenyl cyclase activity (Fig. 6A). Deletion of six N-terminal residues, producing TIP-(7–39), resulted in the loss of detectable ligand-stimulated cAMP accumulation (Fig. 6A). The N-terminal region of TIP39 is therefore a determinant of PTH2 receptor activation. None of the truncated TIP39 analogues detectably activated the PTH1 receptor (Fig. 6B).

In radioligand binding assays, deletion of one, two, four, and six residues from TIP39 results in a progressive reduction of the ligand binding potency for the 293PTH2 receptor (Fig. 7A, Table IV). TIP-(7–39), which does not activate the PTH2 receptor, binds with 70-fold lower affinity to the PTH2 receptor than full-length TIP39 (Fig. 7A, Table IV). (GTPγS (10 µM) reduced binding of 125I-TIP39 to the PTH2 receptor by 62 ± 2%, indicating that the radioligand detects predominantly the receptor-G-protein complex of the PTH2 receptor in these assays.) In contrast, at the PTH1 receptor TIP-(7–39) binds with a 5.6-fold higher affinity than TIP39 (Fig. 7B, Table IV). The N-terminal region of TIP39 is therefore a determinant of TIP39’s selective binding to the PTH2 receptor under conditions in which the G-protein-coupled receptor state is predominantly detected in the binding assay.

The effect of ligand truncation on receptor binding affinity was also measured at the G-protein-uncoupled receptor by measuring ligand binding in the presence of 10 µM GTPγS. Under these conditions, TIP-(7–39) bound to the PTH2 receptor with a 32-fold lower binding potency than full-length TIP39 (Fig. 8A). In contrast, TIP-(7–39) bound with 12-fold higher
affinity to the PTH1 receptor (Fig. 8B). The N-terminal region of TIP39 is therefore a determinant of PTH2/PTH1 receptor binding selectivity at the G-protein-uncoupled receptor state.

In summary, removal of six residues from the N terminus of TIP39 reduces receptor binding affinity at the PTH2 receptor but increases binding affinity at the PTH1 receptor. As a result, the truncation reverses the PTH2/PTH1 receptor binding selectivity of TIP39, such that TIP-(7–39) is a selective, high affinity antagonist of the PTH2 receptor.

### Binding of TIP39 to Chimeric PTH2/Glucagon Receptors—
The PTH2/PTH1 receptor binding selectivity studies above suggest that the juxtamembrane region of the PTH2 receptor contributes strongly to the binding affinity of TIP39. However, the PTH1 receptor binds TIP39 with a moderate affinity and so does not provide a null background in which to measure the contribution of binding interactions to the overall affinity of the ligand. In particular, the PTH2/PTH1 selectivity experiments have not addressed the role of the N-terminal extracellular domain in the binding of TIP39. It is possible that the N-terminal region contributes to the interaction of TIP39 with both PTH2 and PTH1 receptors, but this interaction may not be detected because the selectivity experiments only address the molecular determinants of the difference of ligand affinity between the two receptors.

In order to more directly examine the molecular basis of TIP39 recognition by the PTH2 receptor, we measured TIP39 binding to chimeric PTH2/glucagon receptors. The human glucagon receptor expressed in COS-7 cells did not detectably bind TIP39 at ligand concentrations up to 1 μM (Fig. 9A). This receptor was not detectably activated by TIP39, but glucagon-(1–29) stimulated cAMP accumulation (data not shown), with a 

### Binding of TIP39 to Chimeric PTH2/Glucagon Receptors—

<table>
<thead>
<tr>
<th>Receptor and radioligand</th>
<th>rPTH-(1–34)</th>
<th>TIP39</th>
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<td></td>
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<tr>
<td></td>
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<td>Pseudo-Hill slope</td>
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<td>P2-NP2 125I-TIP39</td>
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<td>1.25 ± 0.26</td>
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</tbody>
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### Parameters for rPTH-(1–34) and TIP39 displacement of radioligand binding to chimeric PTH2/PTH1 receptors and wild-type PTH receptors

Radioligand binding to membranes isolated from COS-7 cells transiently expressing the receptors was measured as described under "Experimental Procedures." Data were fitted to a four-parameter logistic equation to obtain estimates of IC50, Hill slope, and asymptotic maximum and minimum)/(maximum – nonspecific binding) × 100, where nonspecific binding was measured in parallel using a 1.00 μM concentration of the unlabeled analogue of the radioligand. a, significantly different from the TIP39 values for P1 and P1-NP2 (p < 0.001). b, TIP39 values for P1 and P2-NP2 are not significantly different (p > 0.05). c, significantly different from the TIP39 values for P1 and P1-NP2 (p < 0.005). d, TIP39 values for P2 and P2-NP2 are not significantly different (p > 0.05).
DISCUSSION

TIP39 is a good candidate for an endogenous ligand for the PTH2 receptor (1). This peptide ligand has previously been shown to selectively activate the PTH2 receptor and not the closely related PTH1 receptor. In this study, we found that TIP39 also displays binding selectivity for the PTH2 receptor. We have now identified molecular determinants of TIP39 and the PTH receptors that specify the PTH2 receptor selectivity of TIP39 and evaluated the contribution of these regions to the binding affinity of TIP39. The principal findings of this study are as follows. 1) The juxtamembrane domain of the receptor is a determinant of TIP39’s signaling selectivity. 2) The N-terminal region of the ligand is required for PTH2 receptor activation. 3) Ligand binding selectivity is specified by the juxtamembrane domain of the receptor. 4) The N-terminal region of TIP39 is a determinant of ligand binding selectivity; removal of 6 residues from the ligand reverses the PTH2/PTH1 receptor selectivity such that TIP-(7–39) is a selective high affinity antagonist for the PTH1 receptor. 5) A chimeric receptor containing the N-terminal domain of the PTH2 receptor (G-NP2) bound TIP39 (with 55-fold lower affinity than the wild-type receptor) demonstrating that the N-terminal extracellular domain of the PTH2 receptor contributes to TIP39 binding. These findings are consistent with a model in which TIP39 binds with moderate affinity to the N-terminal extracellular domain of the PTH2 receptor, while an interaction between the juxtamembrane domain and N-terminal region of the ligand strongly stabilizes the receptor-ligand complex.

The molecular basis of TIP39’s selective activation of the PTH2 receptor was investigated using chimeric PTH2/PTH1 receptors and truncated TIP39 analogues. The juxtamembrane domain was identified as the principal determinant of signaling selectivity (Fig. 4, Table II). The N-terminal region of TIP39 is required for activation of the PTH2 receptor; TIP-(7–39) did not detectably activate the receptor (Fig. 6A). These findings are consistent with a model in which the N-terminal region of the ligand interacts with the juxtamembrane domain of the receptor leading to receptor activation and second messenger generation. Such an interaction has been proposed for ligand activation for each type II G-protein-coupled receptor for which there are relevant studies (8, 9, 11, 12, 16, 21, 22, 33, 34), including interaction of the human PTH2 receptor with PTH (19, 25). In the evaluation of chimeric PTH2/PTH1 receptors, we found that the juxtamembrane domain did not just support full efficacy ($E_{\text{max}}$) of TIP39; the P2-NP1 chimera was activated by TIP39 with a potency ($-\log EC_{50}$) almost as high as that for the wild-type PTH2 receptor (Fig. 4, Table II). This finding suggested that the juxtamembrane domain of the PTH2 receptor may also contribute to the ligand binding selectivity of TIP39. Measurement of TIP39 binding to chimeric and wild-type receptors confirmed this hypothesis; the juxtamembrane region of the receptor specified the binding selectivity (Fig. 5, Table III).

The data from PTH2/PTH1 chimeric receptor experiments can be used to formulate binding models for TIP39 interaction with the PTH2 and PTH1 receptors. The findings of this study are consistent with the previously proposed two-site model for ligand interaction with type II G-protein coupled receptors; the
TIP39 Interactions with PTH2 and PTH1 Receptors

Binding of truncated TIP39 analogues was measured by displacement of $^{125}$I-TIP39 binding to 293PTH2 membranes and $^{125}$I-[Nle$^{8,21}$,Tyr$^{34}$]rPTH-(1–34) binding to 293PTH1 membranes using the filtration binding assay described under “Experimental Procedures.” Under the conditions of the assay, the receptor-G-protein complex is the predominant receptor state detected. Values were calculated as described for Table III. ND, a maximal inhibition value was not calculated for these experiments because the lower plateau was fixed at nonspecific binding in the curve-fitting analysis.

Table IV. ND, a maximal inhibition value was not calculated for these experiments because the lower plateau was fixed at nonspecific binding in the curve-fitting analysis. *a*, significantly different from TIP39 $-\log IC_{50}$ for the PTH2 receptor ($p < 0.01$); *b*, significantly different from TIP39 $-\log IC_{50}$ for the PTH1 receptor ($p < 0.005$).

<table>
<thead>
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<th>Ligand</th>
<th>PTH2 receptor</th>
<th>PTH1 receptor</th>
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<tbody>
<tr>
<td></td>
<td>$-\log IC_{50}$</td>
<td>Pseudo-Hill slope</td>
</tr>
<tr>
<td>TIP39</td>
<td>8.28 ± 0.30</td>
<td>0.74 ± 0.07</td>
</tr>
<tr>
<td>TIP-(2–39)</td>
<td>7.59 ± 0.33</td>
<td>0.87 ± 0.07</td>
</tr>
<tr>
<td>TIP-(3–39)</td>
<td>7.38 ± 0.13</td>
<td>0.79 ± 0.03</td>
</tr>
<tr>
<td>TIP-(5–39)</td>
<td>6.43 ± 0.28</td>
<td>0.86 ± 0.02</td>
</tr>
<tr>
<td>TIP-(7–39)</td>
<td>6.44 ± 0.23</td>
<td>0.93 ± 0.08</td>
</tr>
</tbody>
</table>

juxtamembrane domain of the PTH2 receptor is required for activation (Fig. 6), and the N-terminal extracellular domain contributes to binding of TIP39, as demonstrated by the G-NP2 PTH2/glucagon receptor chimera (Fig. 9). A description of the model parameters is given in the legend to Fig. 10. Within the framework of this model, two types of interaction could contribute to the PTH2/PTH1 receptor binding selectivity of TIP39. 1) The juxtamembrane domain of the PTH2 receptor may interact with high affinity with the N-terminal region of TIP39 (resulting in a high value of $K_J$ and/or $K_{NJ}$; Fig. 10). 2) The juxtamembrane domain of the PTH1 receptor may be incompatible with the N-terminal region of TIP39, such that this receptor domain acts as an affinity barrier preventing high affinity binding of TIP39 to the PTH1 receptor, reducing the overall affinity of the ligand (low value of $K_J$ and/or $K_{NJ}$). We addressed these possibilities using the TIP39 analogues that had been truncated at the N terminus.

At the PTH2 receptor, TIP-(7–39) does not detectably activate the PTH2 receptor, so it may not appreciably interact with the juxtamembrane domain of the receptor. The postulated weakening of this interaction reduces binding affinity 36- and 70-fold at the R and R-G states, respectively (Figs. 7 and 8), suggesting that an interaction of the N-terminal region of TIP39 with the juxtamembrane domain of the PTH2 receptor contributes strongly to the overall binding energy of the ligand.

Fig. 8. Effect of N-terminal truncation of TIP39 on ligand binding to PTH2 and PTH1 receptors in the presence of 10 μm GTPγS. Binding of TIP39 (○) and TIP-(7–39) (□) was measured by displacement of $^{125}$I-TIP39 binding to 293PTH2 membranes (A) and $^{125}$I-[Nle$^{8,21}$,Tyr$^{34}$]rPTH-(1–34) binding to 293PTH1 membranes (B) using the centrifugation binding assay described under "Experimental Procedures." This assay measures the affinity of ligands for the free receptor, uncoupled from G-protein. Specific binding was defined as in Fig. 5. The mean $-\log IC_{50}$ values for the PTH2 and PTH1 receptors were as follows (IC$_{50}$ values in parentheses): 7.01 ± 0.04 (98 nm) and 8.30 ± 0.05 (5.0 nm), respectively.

Fig. 9. Binding of TIP39 to chimeric PTH2/glucagon receptors and PTH2 and glucagon receptors. A hemagglutinin-tagged PTH2 receptor (P2) (A), the human glucagon receptor (G) (B), a chimeric receptor comprising the N-terminal extracellular domain of the PTH2 receptor and the juxtamembrane region of the glucagon receptor (G-NP2) (C), and the reciprocal chimera (P2-NG) (D) were expressed in COS-7 cells. Binding of TIP39 (○) or human glucagon-(1–29) (○) was measured by displacement of radioligand binding ($^{125}$I-TIP39 for P2 and G-NP2 and $^{125}$I-glucagon for G and P2-NG) using intact cells in 96-well plates, as described under "Experimental Procedures." The total $^{125}$I-TIP39 in A and C was 70,000 cpm, the total $^{125}$I-glucagon in B was 14,000 cpm, and the total $^{125}$I-glucagon in D was 47,000 cpm. Data points are mean ± S.E. of triplicate measurements. The experiments were performed three times with similar results, except for the glucagon receptor for which the experiment was performed twice.
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Fig. 10. Two-site model of receptor-ligand interaction. In this model, two sites on the ligand (A) interact with two corresponding sites on the receptor (R). The C-terminal region of the ligand interacts with the N-terminal extracellular domain of the receptor (forming RA$_2$), defined by the equilibrium association constant K$_{A2}$, and the N-terminal region of the ligand interacts with the receptor juxtamembrane domain (forming RA$_N$, defined by K$_{A_N}$). Following binding either of these domains, ligand binding can be stabilized by interaction with the second domain of the receptor; the stabilization arising from interaction with the juxtamembrane region following binding to the N-terminal domain is defined as K$_{Nj}$, and the reciprocal interaction is defined as K$_{jn}$. The macroaffinity constant of the ligand, K, is equal to (K$_{A2}$ + K$_{A_N}$)$^{1/2} = (K_{Nj} + K_{jn})^{1/2}$.

This binding energy could result from a high value of K$_{A2}$ and/or K$_{A_N}$. We estimate the value of K$_{A2}$ for the R state within this model to be at most 10$^{-7}$ M$^{-1}$ (at least 100 nM as a dissociation constant), since the affinity of TIP39 for the G-NP2 receptor in whole cells is 182 nM and the affinity of TIP (7–39) for the G-protein-uncoupled receptor is 98 nM. The lack of detectable TIP39 binding to the P2-NG receptor chimera suggests that K$_{j}$ may not contribute the additional binding energy, implying that the high affinity interaction of TIP39 results from strong stabilization by interaction with the juxtamembrane region subsequent to interaction with the N-terminal domain (high K$_{Nj}$). However, a structural incompatibility between the PTH2 and glucagon receptors cannot be excluded as an explanation for the lack of TIP39 binding to the P2-NG receptor, so the prediction of a high KNJ value requires further investigation.

In conclusion, we have identified determinants of TIP39’s binding selectivity for the PTH2 receptor and evaluated their contributions to ligand binding affinity. In contrast to the closely related PTH1 receptor, the juxtamembrane domain of the PTH2 receptor and the N-terminal region of TIP39 are determinants of ligand binding selectivity. The binding of TIP39 to the PTH2 receptor is consistent with the two-site model of ligand interaction proposed for the PTH1 receptor, but with the juxtamembrane region of the receptor contributing more strongly to the macroaffinity of the ligand.

REFERENCES
