Differential Contributions of Motilin Receptor Extracellular Domains for Peptide and Non-peptidyl Agonist Binding and Activity*

Received for publication, November 4, 2005, and in revised form, March 9, 2006 Published, JBC Papers in Press, March 10, 2006, DOI 10.1074/jbc.M511921200

Bunzo Matsura1, Maoqing Dong1, Shruthi Naik1, Laurence J. Miller2,1, and Morikazu Onji1,2

From the 1Third Department of Internal Medicine, Ehime University School of Medicine, Shitsukawa 454, Tohon, Ehime 791-0295, Japan and the 2Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, Arizona 85259

The family of G protein-coupled receptors that includes receptors for motilin, ghrelin, and growth hormone secretagogue has substantial potential importance as drug targets. Understanding of the molecular basis of hormone binding and receptor activation should provide insights that are helpful in the development of such drugs. We previously examined the unique second extracellular loop domain of the motilin receptor, identifying key epitopes in perimembranous locations at each end of this long loop (Matsuura, B., Dong, M., and Miller, L. J. (2002) J. Biol. Chem. 277, 9834–9839). Here, we have extended that work, examining the other predicted extracellular domains of the motilin receptor by using sequential deletions of segments ranging from one to six amino acid residues and site-directed alanine replacement mutagenesis approaches. Each construct was transiently expressed in COS cells, and characterized for motilin- and erythromycin-stimulated intracellular calcium responses and motilin radioligand binding. Only those receptor segments that included key Cys residues in positions 25, 30, and 111 or perimembranous regions at the ends of the amino terminus and the first and third extracellular loops disrupted motilin biological activity. Each of these Cys deletions also disrupted action of erythromycin. Alanine replacements for each of the potentially important amino acid residues in the perimembranous segments revealed that residues Gly36, Pro103, Leu109, and Phe332 were responsible for the selective negative impact on motilin biological activity, while responding normally to erythromycin. These results further defined and found to be consistent with regions identified in pho-otic, can act to stimulate receptor activation via interaction with distinct chemical structures, here a peptide and a non-peptidyl antibi-
ocitrate, can act to stimulate receptor activation via interaction with distinct domains. The broad and diffuse base for natural motilin action is also further defined and found to be consistent with regions identified in photosensitivity labeling studies using probes with photolabile sites of covalent attachment in motilin peptide residues 1 and 5 (8, 9). This work also adds insights into key structural features of this receptor, confirming the presence of a disulfide bond that is highly conserved across this superfamily (10, 11) and drawing attention to another likely bond in the amino-terminal tail linking Cys25 and Cys30, thus supporting its functional importance.

EXPERIMENTAL PROCEDURES

Materials—Motilin, erythromycin, and protease-free cell dissociation buffer were from Sigma-Aldrich. Eukaryotic expression vector, pcDNA3.1(−), was from InVitrogen. The human motilin receptor cDNA was kindly provided by Dr. A. D. Howard of Merck Research Laboratories (1). Enzymes used for receptor mutagenesis were purchased from Roche Applied Science or Stratagene (La Jolla, CA). Fura-2/acetoxyethyl ester (Fura-2/AM)3 and Alexa488-conjugated goat

* This work was supported by the Mayo Clinic and Foundation, the Fiterman Foundation, and Grant 16590599, 2004-5 from the Japanese Ministry of Education, Culture, Sports, Science and Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence may be addressed: Dept. of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, 3940 E. Shea Blvd., Scottsdale, AZ 85259. Tel.: 480-301-6650; Fax: 480-301-6969; E-mail: miller@mayo.edu

2 To whom correspondence may be addressed: Ehime University School of Medicine, Shitsukawa 454, Tohon, Ehime 791-0295, Japan. Tel.: 81-89-960-5308; Fax: 81-89-960-5310; E-mail: onjimori@im.ehime-u.ac.jp

3 The abbreviations used are: AM, acetoxymethyl ester; HA, hemagglutinin; PBS, phosphate-buffered saline.
anti-mouse IgG were from Molecular Probes (Eugene, OR). The mouse 12CA5 monoclonal antibody against the hemagglutinin (HA) epitope was from Roche Applied Science.

Receptor Constructs—Motilin receptor constructs were prepared that included segmental deletions of one to six amino acid residues and site-directed alanine replacement mutations in regions predicted to represent the amino-terminal tail and the first and third extracellular loops. These are illustrated in Fig. 1. Constructs were prepared using an oligonucleotide-directed approach. Polymerase chain reactions (PCR) were performed with Pfu Turbo™ DNA polymerase, running 18 cycles of 95 °C for 30 s, 65 °C for 1 min, and 68 °C for 14 min. Products of PCR and restriction enzyme digestions were separated on 1% agarose gels and purified using the Qiagen reagent (Valencia, CA). Receptor constructs were subcloned into the eukaryotic expression vector, pcDNA3.1. Sequences of each of the receptor constructs were confirmed by direct DNA sequencing using an ABI Prism (Foster City, CA) DNA sequencer.

Additionally, a series of HA-tagged constructs were also prepared for immunostaining studies; these included HA-tagged wild-type, alanine site mutants (G36A, P103A, L109A, and P332A), and cysteine deletion mutants (∆25C, ∆30C and ∆111C). They were prepared using the same strategy as described above, by placing the HA sequence (YPYDVP-DYA) at the amino terminus between residues Met¹ and Gly² of the wild type or mutant receptors.

Receptor Expression—Receptor constructs were expressed transiently in COS-1 cells (American Type Culture Collection). In brief, 0.5 × 10⁶ cells plated on tissue culture plasticware were transfected with 3 μg of DNA using a modification of the DEAE-dextran method. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. 72 h after transfection, cells were harvested with cell dissociation medium and used in biological activity and/or radioligand binding assays.

Biological Activity Assays—The ability of each motilin receptor construct to stimulate an intracellular signal in response to motilin and erythromycin was studied using a well established assay for intracellular calcium in Fura-2/AM-loaded transfected COS cells. In this assay, 2.0 × 10⁶ receptor-bearing cells were loaded with 5 μM Fura-2/AM in Krebs-Ringer-HEPES medium (KRH; 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1 mM KH₂PO₄, 2 mM CaCl₂) containing 0.2% bovine serum albumin and 0.01% soybean trypsin inhibitor. Cells were incubated for 20 min at 37 °C. Fluorescence was quantified in a LS55 luminescence spectrometer (PerkinElmer Life Sciences). Excitation was performed at both 340 and 380 nm, with emission determined at 520 nm. Calcium concentrations were calculated from the ratios of these values as described by Grynkiewicz et al. (12). The peak intracellular calcium concentrations achieved were utilized to determine the agonist concentration-dependence of the biological responses. All assays were repeated at least four times.

Receptor Binding Assays—Radioligand binding assays utilized various mutant motilin receptor-bearing cells with a constant amount of radioligand (3–5 pM [¹²⁵I-Ile¹³]-motilin) and varied concentrations of unlabelled motilin (ranging from 0 to 1 μM) in KRH medium containing 0.2% bovine serum albumin and 0.01% soybean trypsin inhibitor. Incubations were carried out for 60 min at 25 °C. The binding assays were performed in 24-well tissue culture plates. Nonspecific binding was determined in the presence of 1 μM motilin and represented less than 20% of total binding. All assays were repeated at least four times.

Immunofluorescence Microscopy—For morphological assessment of receptor expression on the cell surface, COS cells transiently transfected with HA-tagged wild type or mutant motilin receptors were replated to grow on coverslips for 48 h. Cells were washed with PBS and fixed in 2% paraformaldehyde for 30 min. After being washed once with PBS and twice with 1% normal goat serum in PBS, cells were incubated with mouse monoclonal anti-HA antibody

FIGURE 1. Illustration of the human motilin receptor constructs used in this report. Shown is a schematic diagram of the primary sequence and a possible membrane topology (based on hydrophobicity) of the motilin receptor, along with the design of the sequential deletions (in numbered brackets) and Ala replacement constructs (in dark circles with white lettering).
TABLE 1

| Receptor constructs | Motilin binding | Intracellular calcium response to motilin | Intracellular calcium response to erythromycin |
|---------------------|-----------------|------------------------------------------|-----------------------------------------------|
|                     | $K_c$ | $B_{max}$ binding sites/cell | $E_{50}$[motilin] | $E_{max}[Ca^{2+}]$ | $E_{50}$[erythromycin] | $E_{max}[Ca^{2+}]$ |
| WT                  | 1.8 ± 0.3 | 103 ± 25 | 5.4 ± 0.5 | 31 ± 3 | 0.82 ± 0.06 | 45 ± 3 |
| Δ2–4                | 2.9 ± 0.6 | 99 ± 24 | 4.9 ± 0.4 | 30 ± 4 | >100 | 29 ± 8 |
| Δ5–8                | 1.9 ± 0.4 | 101 ± 19 | 5.1 ± 0.3 | 29 ± 3 | >100 | 28 ± 5 |
| Δ9–12               | 3.8 ± 0.8 | 89 ± 16 | 4.2 ± 0.3 | 28 ± 3 | >100 | 25 ± 6 |
| Δ13–16              | 2.6 ± 0.6 | 95 ± 17 | 4.5 ± 0.3 | 28 ± 3 | >100 | 23 ± 3 |
| Δ17–20              | 1.4 ± 0.1 | 111 ± 30 | 4.5 ± 0.2 | 29 ± 3 | >100 | 22 ± 3 |
| Δ21–24              | 2.3 ± 0.4 | 96 ± 18 | 4.0 ± 0.3 | 26 ± 3 | >100 | 21 ± 3 |
| Δ25C                | >1,000 | | >1,000 | 11 ± 2 | >100 | 20 ± 2 |
| Δ26–28              | 1.6 ± 0.3 | 109 ± 20 | 5.1 ± 0.5 | 29 ± 5 | >100 | 19 ± 2 |
| Δ29                 | 8.1 ± 2.4 | 79 ± 18 | 4.2 ± 0.3 | 28 ± 3 | >100 | 18 ± 2 |
| Δ30C                | >1,000 | | >1,000 | 9 ± 1 | >100 | 17 ± 2 |
| Δ31–32              | 2.0 ± 0.3 | 101 ± 32 | 3.7 ± 0.3 | 27 ± 3 | >100 | 16 ± 2 |
| Δ33–36              | >1,000 | | >1,000 | 12 ± 2 | >100 | 15 ± 2 |
| Δ103–104            | >1,000 | | >1,000 | 11 ± 1 | >100 | 14 ± 2 |
| Δ105–110            | >1,000 | | >1,000 | 8 ± 1 | >100 | 13 ± 2 |
| Δ111C               | >1,000 | | >1,000 | 8 ± 2 | >100 | 12 ± 2 |
| Δ328–330            | 6.3 ± 1.9 | 88 ± 26 | 5.1 ± 0.2 | 29 ± 2 | >100 | 11 ± 2 |
| F33A                | 7.9 ± 0.7 | 85 ± 23 | 5.4 ± 0.3 | 29 ± 2 | >100 | 10 ± 2 |
| P34A                | 4.2 ± 1.6 | 90 ± 10 | 4.8 ± 0.3 | 29 ± 2 | >100 | 9 ± 2 |
| L35A                | 3.7 ± 1.1 | 87 ± 16 | 3.9 ± 0.3 | 28 ± 3 | >100 | 8 ± 2 |
| G36A                | >1,000 | | >1,000 | 10 ± 2 | >100 | 7 ± 2 |
| P103A               | >1,000 | | >1,000 | 12 ± 2 | >100 | 6 ± 2 |
| W104A               | 12.2 ± 1.3 | 68 ± 12 | 4.8 ± 0.3 | 29 ± 3 | >100 | 5 ± 2 |
| V105A               | 1.8 ± 0.4 | 109 ± 35 | 4.6 ± 0.3 | 29 ± 3 | >100 | 4 ± 2 |
| F106A               | 1.9 ± 0.5 | 105 ± 26 | 4.5 ± 0.3 | 28 ± 3 | >100 | 3 ± 2 |
| G107A               | 1.0 ± 0.1 | 122 ± 36 | 4.2 ± 0.4 | 27 ± 3 | >100 | 2 ± 2 |
| P108A               | 2.0 ± 0.3 | 97 ± 25 | 4.1 ± 0.4 | 28 ± 4 | >100 | 1 ± 2 |
| L109A               | >1,000 | | >1,000 | 9 ± 2 | >100 | 0 ± 2 |
| L110A               | 2.3 ± 0.5 | 94 ± 15 | 4.5 ± 0.3 | 29 ± 4 | >100 | 0 ± 2 |
| Y331A               | 2.9 ± 0.4 | 83 ± 22 | 4.9 ± 0.4 | 30 ± 4 | >100 | 0 ± 2 |
| F332A               | >1,000 | | >1,000 | 12 ± 1 | >100 | 0 ± 2 |

(1:500) for 1 h. Cells were incubated with Alexa488-conjugated goat anti-mouse IgG (1:200) for 1 h after three washes with 1% normal goat serum in PBS. Coverslips were then washed three times with PBS, mounted on slides, and examined with a Zeiss Axiolux 200M inverted microscope (Carl Zeiss, Oberkochen, Germany) equipped for epifluorescence. All of the above procedures were performed at room temperature.

Statistical Analysis—Biological activity curves and binding curves were analyzed and plotted using the nonlinear regression analysis program in the Prism software package (GraphPad Software, San Diego, CA). Binding kinetics were determined by analysis with the LIGAND program of Munson and Rodbard (13). All observations were expressed as means ± S.E. Data were analyzed using the two-tailed two-way analysis of variance followed by the Newman-Keuls test for multiple comparisons. A value of $p < 0.05$ was considered significant.

RESULTS

Mutagenesis of the Amino Terminus of the Motilin Receptor—In this series of studies, we deleted sequential segments ranging in length from one to four amino acid residues from the predicted amino-terminal tail region of the motilin receptor. These started immediately after the initiator methionine residue and were extended through Gly36 (Fig. 1). Constructs were transiently expressed in COS cells and were characterized functionally (quantitative analysis in Table 1). As shown in Fig. 2A, deletion of only three segments in this region resulted in marked reduction in motilin-stimulated intracellular calcium responses. These included deletion of residues 33 through 36, adjacent to the first transmembrane segment, and deletion of each of the two cysteine residues in this region, Cys25 and Cys30.

To identify potentially important residues in the region between Phe33 and Gly36, each of the relevant four residues was mutated to Ala. Fig. 2B shows that in F33A, P34A, and L35A mutants the intracellular calcium responses to motilin were similar to that in wild-type motilin receptor (quantitative analysis in Table 1). In contrast, the mutation of G36A of the predicted carboxyl-terminal perimembranous end of this extracellular domain resulted in a decreased intracellular calcium response to motilin. The maximal calcium response to motilin for this mutant was only 25% of that for the wild-type motilin receptor.

Shown in Fig. 2, C and D, are the motilin competition-binding curves for each of the deletion (Fig. 2C) and Ala replacement site mutants (Fig. 2D) (quantitative analysis in Table 1). These curves correlated nicely with the activity assays for these constructs (Fig. 2, A and D), with the most dramatic reduction in binding observed for deletion mutants (ΔCys25, ΔCys30, and Δ33–36) and the G36A site mutant.

Mutagenesis of the First Extracellular Loop of the Motilin Receptor—In this region, segments ranging in length from one to six amino acid residues were deleted (Fig. 1). The constructs, representing Δ103–104, Δ105–110, and Δ111C were transiently expressed in COS cells and functionally characterized (quantitative analysis in Table 1). As shown in Fig. 3A, all of the three mutants decreased the intracellular calcium
response relative to that in the wild-type receptor (Fig. 3A). Consistent
with this finding, none of these mutants exhibited detectable binding
(Fig. 3C). These data are consistent with photoaffinity labeling data
using a photolabile motilin analogue that incorporates a benzoylphenyl-
alanine in position 1 (8).

The Cys111 residue that was deleted in the Δ111 construct can be
aligned with a Cys residue that is conserved throughout the superfamily
of G protein-coupled receptors and that contributes to the formation of
the highly conserved disulfide bond linking the first and second extrac-
cellular loops of these receptors. The Cys111 deletion mutant had a
profoundly negative impact (Fig. 3, A and C).

To identify other important residues in this extracellular loop region,
we mutated each of eight residues between Pro103 and Leu110 to Ala (Fig.
3B) and characterized them functionally in COS cells (quantitative anal-
ysis in Table 1). The mutation of P103A within the predicted amino-
terminal perimembranous end of this loop and the mutation of L109A
within the predicted carboxyl-terminal perimembranous end of this
loop resulted in loss of the intracellular calcium responses to motilin.
The maximal calcium response to motilin for these mutants was only
25% of that for the wild-type motilin receptor. Once again, motilin
radioligand binding data correlated with the biological activity studies
(Fig. 3D).
Mutagenesis of the Third Extracellular Loop of the Motilin Receptor—This is a small region, consisting of only five residues. Therefore, only two deletion constructs (Δ328–330 and Δ331–332) were prepared (Fig. 1). They were expressed in COS cells and characterized for biological activity and binding studies (quantitative analysis in Table 1). As shown in Fig. 4A, of the two constructs tested, only the carboxyl-terminal deletion of this loop (Δ331–332) decreased the intracellular calcium response (Fig. 4A) and binding (Fig. 4C). To further explore the importance of individual residues, we mutated Tyr331 and Phe332 to Ala. The mutation of F332A within the predicted carboxyl-terminal end of this loop resulted in loss of intracellular calcium response to motilin (Fig. 4B) and motilin binding (Fig. 4D). This is consistent with our most recent identification of this residue as the site of covalent labeling with a photolabile motilin analogue incorporating a benzoylphenylalanine in position 5 (9).

Evidence of Surface Expression of the Functionally Impaired Mutant Motilin Receptor Constructs—To examine cell surface expression of the functionally impaired motilin receptor constructs as identified above, each of the relevant HA-tagged constructs was transfected into COS cells and immunostained with anti-HA monoclonal antibody. As shown in Fig. 5, each mutant receptor construct was shown to be expressed on the cell surface in density similar to the wild-type receptor. No fluorescence was observed in negative control cells that had been transfected with an empty expression vector or in cells transfected with wild-type receptor but stained with the second antibody only. It should be noted that the HA-tagged motilin receptor had motilin-stimulated biological activity response similar to the wild-type receptor (data not shown).

Biological Responses to Erythromycin—In contrast to the markedly reduced biological responses to motilin for each of the four key alanine site mutants (G36A, P103A, L109A, F332A), these mutants exhibited normal intracellular calcium responses to the non-peptidyl agonist, erythromycin (Fig. 6A, Table 1). Maximal responses were not different for these constructs from values for the wild-type motilin receptor. These support the normal biosynthesis and delivery to the cell surface for each of these important constructs while at the same time supporting differential molecular determinants for peptide and non-peptidyl agonist action.

The constructs that represented the deletion of Cys residues in positions 25, 30, and 111 resulted in decreases in intracellular calcium responses to the natural peptide agonist, motilin, and also resulted in decreased responses to the non-peptidyl agonist, erythromycin (Fig. 6B, Table 1). We previously reported that deletion of Cys235 resulted in reduced intracellular calcium responses to both motilin and erythromycin (7). These findings support the importance of the conserved disulfide bond...
linking Cys$^{111}$ with Cys$^{235}$ in maintaining the receptor architecture important for responses to both peptide and non-peptidyl agonist action.

**DISCUSSION**

The molecular basis of ligand binding to a receptor is dependent on the structural and physicochemical characteristics of both molecules. For the superfamily of G protein-coupled receptors, the heptahelical structure and confluence of these helices in the lipid bilayer are thought to be largely conserved. However, the loop and tail regions are quite varied and likely provide the diversity of themes allowing the binding of structurally diverse natural ligands. This diversity is reflected in different modes of binding for distinct groups of ligands. It is noteworthy that the receptors with structures that are most closely related to each other often have natural ligands that are also structurally related and that bind with similar mechanisms. The motilin receptor is noteworthy because it is structurally similar to the receptors for growth hormone releasing factor and ghrelin. The latter represents the first peptide hormone for which a binding domain at or within the lipid bilayer.

Current insights into the mechanism of binding of motilin have come from receptor mutagenesis and photoaffinity labeling studies (7–9). The only existing mutagenesis studies have carefully examined the predicted second extracellular loop region of the motilin receptor (7). That region was targeted for study because it is quite long in the motilin receptor and much shorter in the ghrelin receptor. The rationale was that this might be critical for the binding of a peptide but less important for the binding of the octanoylated ligand. It turned out that only the regions of importance for peptide binding were the residues at the membrane interface at each end of the long loop (Val$^{179}$, Leu$^{245}$ and Arg$^{246}$) (7). The current report examines all of the other predicted extracellular regions of the motilin receptor following similar experimental strategies of segment deletion and alanine replacement mutagenesis. Here, too, the regions of importance for motilin binding and action were the residues at the membrane interface at the ends of the amino-terminal tail and extracellular loop domains (Gly$^{96}$, Pro$^{103}$, Leu$^{109}$, and Phe$^{332}$). Although all of these regions were critical for the binding and action of motilin, their mutation had no effect on the action of erythromycin, a non-peptidyl agonist acting at this receptor (7).

Of note, this mutagenesis approach also revealed the functional importance of two cysteine residues (Cys$^{55}$ and Cys$^{90}$) within the amino-terminal tail domain that are likely involved in an intradomain disulfide bond within this receptor. Such a bond was not previously suspected to exist. It is particularly interesting that this bond was shown to have functional significance for both motilin and erythromycin action.

Although these data are of great interest and suggest the presence of a platform at the interface between plasma membrane and extracellular domains for natural peptide motilin binding to its receptor, these results are indirect and require complementation. Such loss-of-function studies can be explained by allosteric effects rather than as representing a site of direct ligand interaction with the receptor.

These results are nicely supported by the results of photoaffinity labeling studies in which ligand probes were used that had photolabile benzylophenylalanine sites of covalent attachment in positions one and five (8, 9). The former probe labeled motilin receptor regions between residues 99 and 129, including the first extracellular loop domain, and between residues 130 and 185, including the beginning of the second extracellular loop (8). The latter probe was shown to specifically label Phe$^{332}$ in the third extracellular loop domain (9).

The other interesting and important insight coming from the current work is the clear establishment of distinct mechanisms of binding and action of peptide and non-peptidyl agonists acting at the motilin receptor. Erythromycin is a non-peptidyl motilin receptor agonist that is used therapeutically as a prokinetic agent in motility disorders (14, 15). Disruption of motilin binding and biological activity by mutagenesis of the perimembranous residues in the amino terminus and each of the extracellular loops of this receptor had no negative impact on the action of erythromycin. It is likely that such ligands will bind to intramembranous regions of this receptor. The erythromycin data also represent an important positive control for the normal folding and architecture of the mutant motilin receptors that disrupted motilin binding and action.

In conclusion, we have now demonstrated that residues at each of the predicted perimembranous regions of the amino-terminal tail and extracellular loop domains of the motilin receptor contribute to natural peptide ligand binding and action and are not important for non-peptidyl erythromycin action at this receptor. Additionally, we report evidence for a functionally critical disulfide bond within the amino-terminal tail of this receptor.

**REFERENCES**

1. Feighner, S. D., Tan, C. P., McKee, K. K., Palyha, O. C., Hreniuk, D. L., Pong, S. S., Austin, C. P., Figueroa, D., MacNeil, D., Cassieri, M. A., Nargund, R., Rakshi, R., Abramovitz, M., Stocco, R., Kargman, S., O’Neill, G., Van Der Ploeg, L. H., Evans, J., Patchett, A. A., Smith, R. G., and Howard, A. D. (1999) *Science* **284**, 2184–2188

2. Howard, A. D., Feighner, S. D., Cully, D. F., Arena, J. P., Liberatore, P. A., Rosenblum, C. I., Hamelin, M., Hreniuk, D. L., Palyha, O. C., Anderson, J., Parek, P. S., Diaz, C., Chou, M., Liu, K. K., McKee, K. K., Pong, S. S., Chaung, L. Y., Elbrecht, A., Dashkevich, M., Heavens, R., Rigby, M., Sinnaatansinghij, D. J., Dean, D. C., Melillo, D. G., Patchett, A. A., Nargund, R., Griffin, P. R., DeMartino, J. A., Gupta, S. K., Schaeffer, J. M., Smith, R. G., and Van Der Ploeg, L. H. (1996) *Science* **273**, 974–977

3. Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999) *Nature* **402**, 656–660
4. McKee, K. K., Tan, C. P., Palyha, O. C., Liu, J., Feighner, S. D., Hreniuk, D. L., Smith, R. G., Howard, A. D., and Van der Ploeg, L. H. (1997) Genomics 46, 426–434
5. Hosoda, H., Kojima, M., Matsuo, H., and Kangawa, K. (2000) Biochem. Biophys. Res. Commun. 279, 909–913
6. Hosoda, H., Kojima, M., Matsuo, H., and Kangawa, K. (2000) J. Biol. Chem. 275, 21995–22000
7. Matsuura, B., Dong, M., and Miller, L. J. (2002) J. Biol. Chem. 277, 9834–9839
8. Coulie, B., Matsuura, B., Dong, M., Hadac, E. M., Pinon, D. I., Feighner, S. D., Howard, A. D., and Miller, L. J. (2001) J. Biol. Chem. 276, 35518–35522
9. Matsuura, B., Dong, M., Coulie, B., Pinon, D. I., and Miller, L. J. (2005) J. Pharmacol. Exp. Ther. 313, 1101–1108
10. Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. (1994) Annu. Rev. Biochem. 63, 101–132
11. Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., and Sealfon, S. C. (1992) DNA Cell Biol. 11, 1–20
12. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
13. Munson, P. J., and Rodbard, D. (1980) Anal. Biochem. 107, 220–239
14. Peeters, T. L. (1993) Gastroenterology 105, 1886–1899
15. Janssens, J., Peeters, T. L., Vantrappen, G., Tack, J., Urbain, J. L., De Roo, M., Muls, E., and Bouillon, R. (1990) N. Engl. J. Med. 322, 1028–1031