Differential Determinants for Peptide and Non-peptidyl Ligand Binding to the Motilin Receptor

CRITICAL ROLE OF SECOND EXTRACELLULAR LOOP FOR PEPTIDE BINDING AND ACTION*

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The predicted second extracellular loop domain of the motilin receptor is of particular interest because it is a region that is quite distinct from the analogous regions in other family members that are most closely related and because the initial report of the photoaffinity labeling of a domain of this receptor included this region (Coulie, B. J., Matsuura, B., Dong, M., Hadac, E. M., Pinon, D. L., Feighner, S. D., Howard, A. D., and Miller, L. J. (2001) J. Biol. Chem. 276, 35518–35522). In the current work, motilin receptor constructs were prepared that included sequential deletions ranging from single residues to twelve amino acid segments throughout this 67 amino acid domain. Each construct was expressed in COS cells and characterized for motilin radioligand binding and motilin-stimulated intracellular calcium responses. The only segments that had negative impact on motilin binding and biological activity included deletion constructs ΔCys235, 179–182, and 241–246. Cys235 is likely involved in the highly conserved and functionally important disulfide bond linking the first and second loops of G protein-coupled receptors. Alanine replacements for each of the amino acid residues in the other two segments revealed that the perimembranous residues at both ends of this loop, Val179 and Leu245 and Arg246, were responsible for the negative impact on motilin binding and biological activity. Of note, these mutants responded normally to the non-peptidyl agonist, erythromycin. These data support important functional roles for both amino-terminal and carboxyl-terminal perimembranous regions of the second loop for responses to the natural agonist peptide, while supporting independent determinants for action of a non-peptidyl agonist ligand.

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The superfamily of guanine nucleotide-binding protein (G protein)-coupled receptors is remarkable for its size and diversity, having the largest number of receptors and the greatest variation in natural ligands. These receptors have been organized into classes that share major structural themes, and into smaller groups called families that share a finer level of structural similarities (2). It is noteworthy that receptors in a single family often also have natural ligands that share structural similarities with each other as well. It has been assumed that these ligands bind to the receptors in their family with similar themes.

It was recently established that the motilin receptor and growth hormone secretagogue receptors comprise a new family within class I G protein-coupled receptors (3–5). As might be expected, when a natural ligand for the growth hormone secretagogue receptor was subsequently identified as ghrelin, it had significant primary sequence homology with the motilin peptide (6, 7). However, both motilin and ghrelin have unique structural features that make the molecular basis of receptor binding and activation of these receptors particularly interesting.

This relates to the requirement in ghrelin for the novel post-translational modification of N-octanoylation of the Ser residue in its third position (6). No analogous modification of motilin peptides has been reported. Further, motilin has an unusual structure-activity relationship for a peptide receptor in the class I family of G protein-coupled receptors, having amino-terminal rather than the predominantly carboxyl-terminal sequence determinants for its selectivity of binding and action (3, 8).

Despite the clear sequence homology between motilin and growth hormone secretagogue receptor sequences, particularly in their predicted transmembrane segments (86%), there is a prominent difference in the region predicted to represent the second extracellular loop domain (Fig. 1). This 67-amino acid loop within the motilin receptor is 40 residues longer than the analogous loop in the growth hormone secretagogue receptor. However, whether these additional residues are functionally significant is not yet clear.

In the current work, we focused on the second extracellular loop domain of the motilin receptor and used site-directed mutagenesis with sequential deletion of segments and alanine-scanning mutagenesis to identify functionally important residues. Of note, both ends of this loop, representing domains that are conserved throughout this family, were found to be functionally important for the binding and action of the natural peptide agonist, motilin, whereas the nonconserved residues in the mid-region of the loop were not necessary. Additionally, those regions that were important for peptide responses were not critical for responses to a non-peptidyl agonist known to act at this receptor, erythromycin, supporting distinct receptor structural determinants for action of these two chemically distinct agonist ligands. Although disruption of the conserved disulfide bond that links this extracellular loop to the first extracellular loop domain of this receptor by deletion of a component cysteine (Cys235) completely eliminated calcium signal-
ing in response to the natural peptide agonist, motilin, erythro-romycin continued to elicit a response in this construct. This finding further supported the distinct structural basis for the action of peptide and non-peptidyl agonist ligands of this receptor.

**EXPERIMENTAL PROCEDURES**

**Materials—**Enzymes used for receptor mutagenesis were purchased from Roche Molecular Biochemicals except for *Pfu Turbo* DNA polymerase, which was from Stratagene (La Jolla, CA). The solid-phase oxidant, N-chlorosuccinimide/sulfonamide (Iodo-bead), was from Pierce. Fura-2/AM was from Molecular Probes (Eugene, OR). 2-(Trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). All other reagents were analytical grade.

**Peptides—**Human motilin and its oxidation-resistant analogue used for radiolabeling, [Ile13]motilin, were synthesized using manual solid-phase techniques (1). Both peptides were purified to homogeneity by reversed-phase HPLC, with the chemical identities of products established by mass spectrometry. [Ile13]Motilin was radiolabeled oxidatively with Na[125I]I using a 15-s exposure to an Iodo-bead (Pierce) and purifying the product by reversed-phase HPLC to yield specific radioactivity of 2,000 Ci/mmol (9).

**Receptor Constructs—**The human motilin receptor cDNA was kindly provided by Dr. Andrew D. Howard of Merck Research Laboratories (Rahway, NJ) (3). A series of mutations of the motilin receptor second extracellular loop domain was prepared (Fig. 1). These represented deletions of segments ranging in length from 1 to 12 amino acid residues and substitutions of single amino acid residues in domains of interest with Ala. Mutant receptors were constructed using an oligonucleotide-directed approach with the QuikChange Site-Directed Mutagenesis Kit from Stratagene. PCR incubations were performed with 3% (v/v) dimethyl sulfoxide in a thermal cycler 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 the PCR and restriction enzyme digestion were separated on 1% agarose gels and purified using the Qiagen polymerase, running 18 cycles of 95 °C for 14 min. The sequences of all constructs were confirmed by direct DNA sequencing (10).

**Receptor Expression—**Receptor constructs were expressed transiently in COS-1 cells (American Type Culture Collection). For this, 0.5 × 106 cells plated on tissue culture plasticware were transfected with 3 μg of DNA using a modification of the DEAE-dextran protocol that included dimethyl sulfoxide shock and treatment with 0.1 mM chloroquine diphosphate (11). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% Fetal Clone II (Hyclone Laboratories, Logan, UT). Cells were harvested mechanically 72 h after transfection for radioligand binding and biological activity assays.

**Receptor Binding Assay—**Plasma membranes from receptor-bearing cells were prepared using a procedure that included cell disruption by Deounce homogenization and sucrose gradient ultracentrifugation (12). In the standard binding assay, enriched membranes (5–10 μg of protein) were incubated with a constant amount of radioligand, [125I]Ile13-motilin (3–5 pm), in the presence of increasing concentrations of unlabeled motilin (ranging from 0 to 1 μM) for 1 h at room temperature (1). Incubations were performed in Krebs-Ringer-HEPES medium containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KHPO4, 1.2 mM MgSO4, 2 mM CaCl2, 0.01% soybean trypsin inhibitor, and 0.2% bovine serum albumin. After reaching steady state, bound and free radioligand were separated using a Skatron cell harvester (Molecular Devices, Sunnyvale, CA) with receptor-binding filter mats; bound radioactivity was quantified in a γ-spectrometer. Nonspecific binding, defined as radioligand bound in the presence of 1 μM unlabeled motilin, represented less than 20% of total radioligand bound. 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).

For selected experiments, motilin radioligand binding was performed using cells that were not treated for 30 min at 37 °C with a cell-impermeant reductant (10 mM glutathione) and/or a cell impermeant sulfhydryl-reactive methanethiosulfonate reagent (5 mM MTSET). Bound and free radioligand were separated by centrifugation and washing. All assays were set up to include duplicates of each condition, with assays repeated a minimum of three independent times.

**Intracellular Calcium Biological Activity Assay—**The ability of each motilin receptor construct to transmit an intracellular signal in response to motilin was studied using a well established assay for intracellular calcium in Fura-2/AM-loaded transfected COS cells. In this assay, 2.0 × 105 receptor-bearing cells were loaded with 5 μM Fura-2/AM (Molecular Probes) in calcium-free Krebs-Ringer-HEPES medium for 20 min at 37 °C. After washing, cells were stimulated with varied concentrations of motilin or erythromycin at 37 °C. Fluorescence was quantified in a PerkinElmer LS50B luminescence spectrometer. Excitation was performed at both 340 and 380 nm, with emission determined at 520 nm. Calcium concentration was calculated from the ratios of these values as described by Grynkiewicz et al. (14). The peak intracellular calcium concentration that was transiently achieved was utilized to determine the agonist concentration dependence of the biological responses. All assays were repeated at least four times.

**RESULTS**

**Motilin Receptor Structure—**The primary sequence of the human motilin receptor, established in 1999 (3), was found to be most similar to the sequences of the receptors for growth hormone secretagogues (4, 5). Together, these represent a closely related family within class I G protein-coupled receptors. It is noteworthy, however, that the receptor for motilin includes a long segment (40 amino acid residues) inserted in its predicted second extracellular loop domain that is not present in the growth hormone secretagogue receptor (Fig. 1B). Ghrelin...
Ligand-binding Domain of Motilin Receptor

**FIG. 2.** Binding and biological activities of the motilin receptor second extracellular loop deletion constructs. **Left column,** results from competition-binding experiments utilizing enriched plasma membranes from COS cells transfected with wild type motilin receptor and with each of the second loop deletion constructs. All curves represent homologous competition of increasing concentrations of unlabeled motilin for the binding of a constant amount of the motilin radioligand, 125I-[Ile13]motilin. Values illustrated represent saturable binding as a percentage of the control situation for binding to cell membranes expressing wild type motilin receptor in the absence of competing motilin. Data points represent the means ± S.E. of three experiments. **Right column,** intracellular calcium responses to motilin in the COS cells transfected with each of these receptor constructs. Values are expressed as the means ± S.E. of data from four assays, normalized relative to the maximal response to motilin in cells expressing the wild type receptor.

**TABLE I**
Quantitative analysis of binding and biological activity data

Shown are the parameters of motilin radioligand binding to COS cell membranes expressing each of the noted motilin receptor constructs that were determined using the LIGAND program of Munson and Rodbard (13). Also shown are the concentrations of motilin able to elicit a half-maximal intracellular calcium response in these cells. WT, wild type.

| Receptor constructs | Motilin binding, $K_d$ (nM) | Motilin binding, $B_{max}$ (pmol/mg) | Intracellular calcium response, $EC_{50}$ (nM) |
|---------------------|-----------------------------|-------------------------------------|-------------------------------------|
| WT                  | 4.4 ± 2.9                   | 49 ± 24                             | 1.1 ± 0.1                           |
| Δ179–182            | >1,000                      | >1,000                              | >1,000                              |
| Δ183–186            | 7.5 ± 1.9                   | 34 ± 5                              | 1.9 ± 0.1                           |
| Δ187–190            | 5.5 ± 0.5                   | 29 ± 11                             | 1.9 ± 0.1                           |
| Δ191–202            | 23 ± 9                      | 12 ± 9                              | 1.7 ± 0.1                           |
| Δ203–214            | 6.4 ± 5.0                   | 15 ± 14                             | 1.7 ± 0.1                           |
| Δ215–228            | 19 ± 10                     | 29 ± 20                             | 1.7 ± 0.1                           |
| Δ227–234            | >1,000                      | >1,000                              | >1,000                              |
| ΔCys235             | >1,000                      | 5.0 ± 2.0                           | 1.5 ± 0.1                           |
| Δ236–240            | 3.7 ± 1.5                   | 3.0 ± 1.2                           | >1,000                              |
| Δ241–246            | >1,000                      | >1,000                              | >1,000                              |
| V179A               | >1,000                      | >1,000                              | >1,000                              |
| E190A               | 15 ± 7                      | 20 ± 11                             | 1.4 ± 0.1                           |
| Q181A               | 53 ± 10                     | 67 ± 16                             | 1.5 ± 0.1                           |
| D182A               | 29 ± 4                      | 30 ± 12                             | 2.6 ± 0.4                           |
| Q241A               | 6.3 ± 5.0                   | 8 ± 6                               | 2.2 ± 0.2                           |
| L242A               | 6.7 ± 3.0                   | 12 ± 5                              | 2.5 ± 0.3                           |
| G243A               | 4.5 ± 2.0                   | 19 ± 15                             | 1.6 ± 0.2                           |
| L245A               | >1,000                      | >1,000                              | >1,000                              |
| R246A               | >1,000                      | >1,000                              | >1,000                              |

has been identified as a natural agonist ligand for the growth hormone secretagogue receptor (6). It is a 28-residue peptide that has 8 of the 22 residues within motilin in analogous positions (motilin residues Phe6, Glu9, Gln11, Arg12, Gln14, Lys20, Glu22, and Lys25), but it also has a critically important post-translational modification of the Ser residue in the third position, N-octanoylation (6), that has not been observed for motilin peptides (Fig. 1C).

**Deletional Mutagenesis of the Second Extracellular Loop of the Motilin Receptor**—In this series of studies, we deleted sequential segments from the predicted beginning of the second extracellular loop domain (Val179) to the end of this domain (Arg246). These constructs were transiently expressed in COS cells and functionally characterized. Fig. 2, **Left column** shows the motilin competition-binding curves for each of these constructs (quantitative analysis in Table I). Segments ranging in length from 4 to 12 amino acid residues were deleted. These demonstrated only three regions in this large second loop domain that had substantial impact on motilin binding. These included the deletion constructs Δ179–182 and Δ241–246 sited at the amino-terminal and carboxyl-terminal ends of this loop, as well as ΔCys235.

Shown in Fig. 2, **Right column** are the biological activity curves for intracellular calcium responses to motilin stimulation for each of the receptor deletion constructs (Table I). These curves correlated nicely with the radioligand binding data, with the most dramatic reductions in motilin-stimulated sig-
naling observed for each of the three critical domains described above. Also observed were quantitatively small reductions in intracellular calcium responses for deletions between residue 187 and 240.

Site-directed Mutagenesis of the Amino-terminal Perimembranous Region of the Second Loop Domain—To identify potentially important residues in the region between Val179 and Asp182, we mutated each of these four residues to Ala. Fig. 3, left column shows that the D182A, Q181A, E180A mutants bound to motilin with affinities similar to that of the wild type motilin receptor. In contrast, mutation of Val179 to Ala resulted in loss of demonstrable, saturable motilin radioligand binding. Correspondingly, the biological activity of these mutants in response to motilin decreased as the site of mutation moved toward the amino-terminal perimembranous end of this loop domain (Fig. 3, right column). The maximal calcium response to motilin for the V179A receptor construct was only 25% of that for the wild type motilin receptor.

Site-directed Mutagenesis of the Carboxyl-terminal Perimembranous Region of the Second Loop Domain—We also mutated each of the residues in the region between Gln241 and Arg246 to Ala to further explore their individual importance. Fig. 4, left column shows that mutants Q241A, L242A, and G243A each bound motilin radioligand binding. Correspondingly, the biological activity of these mutants in response to motilin decreased as the site of mutation moved toward the carboxyl-terminal perimembranous end of this loop domain (Fig. 4, right column). Maximal calcium responses to motilin for both L245A and R246A receptor constructs were less than 40% of that for the wild type motilin receptor.

Action of a Non-peptidyl Agonist Ligand—in contrast to the markedly reduced biological responses to motilin for each of the three key alanine site mutants (V179A, L245A, and R246A), these mutants exhibited normal intracellular calcium responses to the non-peptidyl agonist erythromycin (Fig. 5). Maximal responses and EC50 values were not different for these constructs from values for the wild type motilin receptor.

Region of a Conserved Disulfide Bond—The Cys235 residue that was deleted in the ΔCys235 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 that links the first and second extracellular loops of these receptors. The deletions around this residue had no impact on motilin binding or motilin-stimulated intracellular calcium responses, whereas deleting Cys\textsubscript{235} had a profoundly negative impact (Fig. 2). This likely reflects the involvement of this residue in a disulfide bond that, by analogy with other G protein-coupled receptors, helps to establish the global architecture of the helical bundle. Of note, when the correctly folded motilin receptor on the surface of the cell was exposed to the cell-impermeant reductant, glutathione, in the absence or presence of chemical modification with the highly reactive methanethiosulfonate reagent MTSET, there was no negative impact on motilin binding (Fig. 6). The same treatment (reduction and/or methanethiosulfonate reagent derivatization of exposed sulfhydryl groups) of intact cells expressing the secretin receptor known to have functionally critical extracellular disulfide bonds resulted in a disruption of secretin binding (positive controls) (15). This suggests that the fully folded motilin receptor can utilize other intramolecular interactions to maintain its appropriate global conformation.

Further evidence for differential determinants for the action of peptide and non-peptidyl agonists came from stimulation of the \(\Delta\text{Cys}^{235}\) construct with erythromycin (Fig. 5). Although the potency of this agonist to stimulate an intracellular calcium response was normal, its efficacy was reduced by approximately half. This finding is in direct contrast with the inability of the natural peptide agonist, motilin, to stimulate this signaling event in this construct (Fig. 2). It is likely that the efficiency in establishing the correct confluence of transmembrane helical domains in this construct is lower than normal, supporting binding and signaling in the intact helical bundles, whereas the additional domains important for peptide action are even less likely to fold properly.

**DISCUSSION**

The motilin-growth hormone secretagogue family of receptors may follow unique and interesting molecular themes for binding and being activated by their natural ligands. This relates to the location of critical determinants for binding and biological activity at the amino-terminal ends of both motilin (3, 8) and ghrelin (6) rather than at the carboxyl terminus, which is most typical of peptide receptors within the class I G protein-coupled receptors (2). It is also possible that the members of this receptor family follow themes that are distinct even among themselves. This relates to the novel and functionally critical post-translational modification of ghrelin, N-octanoylation of Ser\(^3\) (6), which is apparently absent in motilin. Such a modification could establish an association with the lipid bilayer that orients ghrelin in a unique manner prior to its receptor binding.

To explore the possibility of distinct modes of binding for members of this receptor family, we focused our efforts on a domain of substantial difference between the motilin and growth hormone secretagogue receptors in the second extracellular loop domain. This region includes a 40-amino acid insertion in the motilin receptor that is absent in the growth hormone secretagogue receptor. The potential importance of this unique region was further emphasized by the recent report of the first photoaffinity labeling of a motilin receptor domain that included this loop (1).

We followed the classical experimental approaches of deletional and alanine-scanning mutagenesis. The results of these efforts focused interest on three regions within the second extracellular loop. A key amino acid is Cys\textsubscript{235}, a highly conserved residue that contributes to a conserved disulfide bond linking the first and second extracellular loop domains of essentially every member of the G protein-coupled receptor superfamily. Deletions on both sides of this residue had no negative impact, whereas deletion of only this residue had a profound negative effect on receptor biosynthesis and its ability to bind and signal in response to motilin. Even micromolar concentrations of motilin elicited no intracellular calcium response from cells expressing this construct. In contrast, and of particular interest, cells expressing this construct were still able to respond to the non-peptidyl agonist, erythromycin. This represented a clear indication that non-peptidyl agonist had receptor structural determinants for binding and action that were clearly distinct from those for the natural peptide agonist ligand.

The second region of interest represented the amino-terminal perimembranous domain of the second loop. Here, Val\textsubscript{179} was shown to be responsible for the negative impact of the mutants on motilin responses. There was evidence that the V179A construct is synthesized normally and undergoes normal trafficking to the cell surface, because there was a small biological response to the cell-impermeant peptide ligand, motilin, even though its affinity was too low to detect by radioligand binding assay. This negative impact on motilin binding and biological activity could be either direct, interacting with...
the bound peptide ligand, or indirect, acting via an allosteric effect on receptor conformation. Because this hydrophobic amino acid is predicted to reside at or near the interface with the lipid bilayer, many potentially important allosteric effects can be proposed. An important control demonstrated normal responsiveness to erythromycin. This likely reflects the different receptor domains necessary for the action of peptide and non-peptidyl agonist ligands.

The third region of interest represented the carboxyl-terminal perimembranous domain of the second loop. Here, both Leu\textsuperscript{245} and Arg\textsuperscript{246} appear to contribute to the negative impact of the nal perimembranous domain of the second loop. Here, both non-peptidyl agonist ligands, ent receptor domains necessary for the action of peptide and responsiveness to erythromycin. This likely reflects the different receptor domains necessary for the action of peptide and non-peptidyl agonist ligands.

The same types of considerations described above for the amino-terminal perimembranous domain are relevant here as well. Both of these domains would be expected to be spatially approximated with each other in the intact, fully folded receptor molecule. At the present time, there is no credible model to illustrate this, although the constraint contributed by the current support for the disulfide bond linking the first and second loop domains will provide a starting point for developing such a model.

These observations indeed support the functional importance of the motilin receptor second extracellular loop region for binding and activation by the natural peptide ligand. Perhaps surprisingly, the structural feature of this loop that was most responsible for focusing our attention in these studies, the 40-amino acid residue insertion not present in the growth hormone secretagogue receptor, appears to play no functional role. Instead, the functionally important domains within this extracellular loop are all conserved in the other structurally related G protein-coupled receptors. These include the highly conserved disulfide bond that almost certainly has a global architectural role in the superfamily and also both extreme perimembranous regions of this loop. The precise functional roles for peptide binding and action for the identified key residues remain to be defined.

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