Identification of Peptide Ligand-binding Domains within the Human Motilin Receptor Using Photoaffinity Labeling*

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Barnard Coulie, Bunzo Matsuura, Maoting Dong, Elizabeth M. Hadac, Delia I. Pinon, Scott D. Feighner, Andrew D. Howard, and Laurence J. Miller

From the Center for Basic Research in Digestive Diseases, Departments of Internal Medicine and Biochemistry/Molecular Biology, Mayo Clinic and Foundation, Rochester, Minnesota 55905 and the Department of Metabolic Disorders, Merck Research Laboratories, Rahway, New Jersey 07065

The cDNA encoding the human motilin receptor was recently cloned and found to represent a G protein-coupled receptor that is structurally related to the growth hormone secretagogue receptors. Together, these represent a new Class I receptor family. Our aim in the present work is to gain insight into the molecular basis of binding of motilin to its receptor using photoaffinity labeling. To achieve this, we developed a Chinese hamster ovary cell line that overexpressed functional motilin receptor (CHO-MtlR; 175,000 sites per cell, with $K_i = 2.3 \pm 0.4 \text{ nM}$ motilin and $EC_{50} = 0.3 \pm 0.1 \text{ nM}$ motilin) and a radiiodinatable peptide analogue of human motilin that incorporated a photolabile $p$-benzoyl-L-phenylalanine (Bpa) residue into its pharmacophoric domain. This probe, [Bpa]$^1$Ile$^{13}$motilin, was a full agonist at the motilin receptor that increased intracellular calcium in a concentration-dependent manner ($EC_{50} = 1.5 \pm 0.4 \text{ nM}$). This photolabile ligand bound specifically and with high affinity to the motilin receptor ($K_i = 12.4 \pm 1.0 \text{ nM}$), and covalently labeled that molecule within its $M_r = 45,000$ deglycosylated core. Cyanogen bromide cleavage demonstrated its covalent attachment to fragments of the receptor having apparent $M_r = 6,000$ and $M_r = 31,000$. These were demonstrated to represent fragments that included both the first and the large second extracellular loop domains, with the latter representing a unique structural feature of this receptor. The spatial approximation of the pharmacophoric domain of motilin with these receptor domains support their functional importance as well.

Motilin has long been recognized as an important endogenous peptide regulator of gastrointestinal motor function (1, 2). The receptor for this hormone has also been demonstrated to represent a clinically useful pharmacological target, activated by erythromycin (3, 4). While there have been extensive physiological and pharmacological studies of motilin action, including extensive analysis of peptide structure-activity relationships (5–7), the motilin receptor (MtIR)$^1$ has been difficult to isolate and biochemically characterize.

Using a unique high throughput screen of compounds that can interact with cloned orphan receptors, the cDNA encoding the human motilin receptor (originally isolated as orphan clone GPR38) was finally identified in 1999 (8). Sequence analysis demonstrated that this represents a member of a new subgroup within the Class I family of G protein-coupled receptors that is defined by structural homology with a series of growth hormone secretagogue receptors (9, 10). The motilin receptor is 52% identical to the human growth hormone secretagogue receptor, with 86% sequence identity in the predicted transmembrane domains (8, 10). Because this group of receptors has only been recognized for a short time, no specific receptor domains of importance for binding or activation have yet been identified. Better understanding of the molecular basis of activation of this receptor by its natural ligand may provide important insights for drug development.

Many existing structure-activity studies have focused on the relationship between the structure of motilin, the natural peptide ligand, and its ability to bind to or to stimulate contractile activity of natural motilin receptor-bearing gastrointestinal smooth muscle tissue (5–7, 11, 12). These studies have demonstrated that the amino-terminal portion of motilin, including residues 1–9, is devoid of any activity, while extension of this domain beyond the first nine residues restores binding and biological activity (6, 8). Such observations have established that the pharmacophoric domain of this hormone represents its amino-terminal decapeptide. The carboxyl-terminal region of motilin forms an $\alpha$-helix that is thought to stabilize the interaction of the critical amino-terminal residues at the active site of the receptor (7).

In this work, we have attempted to develop the tools necessary to begin to gain an understanding of the molecular basis of motilin binding to its receptor. This included the establishment of a cell line that expresses a high density of functional motilin receptors and the development of a radiiodinatable motilin analogue that incorporates a photolabile residue within this critical pharmacophoric domain of the natural peptide ligand. In the present work, we have developed and fully characterized these reagents and have applied them to the photoaffinity labeling of the motilin receptor and to the initial identification of distinct subdomains within that molecule. The [Bpa]$^1$Ile$^{13}$motilin probe was shown to covalently label the motilin receptor within two cyanogen bromide fragments, with one including its large second extracellular loop, a structurally unique and functionally important region.

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† These authors contributed equally to this work.

¶ To whom correspondence should be addressed: Center for Basic Research in Digestive Diseases, Guggenheim 17, Mayo Clinic, 200 First St, SW, Rochester, MN 55905. Tel.: 507-284-0680; Fax: 507-284-0762; E-mail: miller@mayo.edu.

1 The abbreviations used are: MtIR, motilin receptor; CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography; Bpa, $p$-benzoyl-L-phenylalanine; Boc, $t$-butoxycarbonyl; MES, 4-morpholineethanesulfonic acid.
Ligand-binding Domain of the Motilin Receptor

molitin-1-22
F-V-P-P-T-T-Y-R-E-L-G-Q-M-E-K-E-R-N-K-G-Q

[^125I](Ile^{13})molitin-1-22
F-V-P-P-T-T-Y-R-E-L-G-Q-M-E-K-E-R-N-K-G-Q

[^125I](Bpa^{1},Ile^{13})molitin-1-22
Bpa-V-P-P-T-T-Y-R-E-L-G-Q-M-E-K-E-R-N-K-G-Q

Fig. 1. Synthetic Peptides. Shown are the sequences of natural human motilin-(1-22), as well as synthetic analogues of this peptide used for radioligand binding studies and photoaffinity labeling studies. Modifications of residues found in the natural peptide are highlighted. Peptides were radioiodinated oxidatively on the Tyr residue in position 7.

Materials and Methods

Reagents—The solid phase oxidant, N-chlorobenzenesulfonamide (Iodo-beads), was from Pierce Chemical Co. Endoglycosidase F was prepared in our laboratory, as we have reported (13). Fura-2AM was from Molecular Probes (Eugene, OR). Other reagents were analytical grade. The wild type human motilin receptor cDNA was kindly provided by Merck Research Laboratories, Rahway, NJ.

Receptor Preparations—We established a Chinese hamster ovary (CHO) cell line stably expressing the wild type human motilin receptor (CHO-MtIR) by transfecting CHO-K1 cells with the motilin receptor cDNA construct. A clonal cell line was selected by repeated cycles of limiting dilution, screening for motilin radioligand binding (as described below). This cell line was used as a source of receptor for the current study. Cells were cultured at 37 °C on tissue culture plasticware in Ham’s F-12 medium supplemented with 5% Fetal Clone-2 (HyClone Laboratories, Logan, UT). Cells were passaged twice a week and were lifted mechanically after incubation in cell dissociation medium before membrane preparation or study of intact cells. Enriched plasma membranes from these cell lines were prepared as previously described (14).

Mutant motilin receptor constructs were prepared using an oligonucleotide-directed approach (QuickChange site-directed mutagenesis system, Stratagene). Two such constructs were prepared for this work, representing replacement of motilin receptor residues Leu^{10} and Ile^{13} with Met residues (L98M and I185M motilin receptors). These residues were in positions predicted to reside in the second transmembrane segment and in the second extracellular loop domain. Sequences of these constructs were confirmed by direct DNA sequencing (15). These receptor constructs were expressed transiently on COS cells after transfection using a modification of the DEAE-dextran method (16). Sixty hours after transfection, cells were lifted mechanically, and enriched plasma membranes were prepared using the method previously described (14).

Synthesis of Peptides—Human motilin-(1-22) (motilin), [Ile^{13}]motilin, and [Bpa^{1},Ile^{13}]motilin peptides (Fig. 1) were synthesized by manual solid-phase techniques using Boc-protected amino acids, hydroxybenzotriazol, and Pamo acid. t-Boc-Ile^{13}Bpa was prepared as described (17, 18). The synthetic products were purified to homogeneity by sequential reversed-phase HPLC separations (19). Identities of the peptides were determined by quantitative amino acid analysis and mass spectrometry. t- and d-Bpa stereoisomers were identified using the technique of Miller and Kaiser (20), involving the full cleavage of the peptide containing the L form and limited or absent cleavage of the peptide containing the D form with amino peptidase M. The t-Bpa peptide was used in the current studies.

Radioiodination of Peptides—[Ile^{13}]motilin and [Bpa^{1},Ile^{13}]motilin peptides were radioiodinated oxidatively on Tyr residues in position 7 of each peptide using Na[^125I] and a 15 s exposure to the solid phase oxidant, N-chlorobenzenesulfonamide (Iodo-beads). The products were purified to homogeneity by reversed-phase HPLC to yield specific radioactivities of 2,000 Ci/mmol (19).

Biological Activity Determination—The agonist activities of motilin and [Bpa^{1},Ile^{13}]motilin were studied using an assay for stimulation of the agonist concentration dependence of the biological responses. All assays were performed in duplicate and repeated at least three times in independent experiments.

Receptor Binding Studies—Radioligand binding assays utilized intact motilin receptor-bearing cells or an enriched plasma membrane fraction of these cells (1-10 µg per tube), [^125I](Ile^{13})motilin (3-5 µCi radioligand) and KRB medium. Incubations were for 60 min at 25 °C. The intact cell binding assay was performed in a 24-well tissue culture plate, while the membrane binding assay utilized separation of bound and free radioligand using the Vitatron apparatus (Molecular Devices, Sunnyvale, CA) and receptor-binding filtermats. Nonspecific binding was determined in the presence of 1 µM motilin and represented less than 20% of total binding.

Photoaffinity Labeling of the Motilin Receptor—Photoaffinity labeling was performed using enriched plasma membrane preparations (100 µg per tube) and the radioiodinated photolabile analogue of motifin described above (100 µCi). Incubations were performed for 60 min in the dark at 25 °C. After binding, membranes were exposed to photolysis for 30 min at 4 °C using a Rayonet Photochemical Reactor (Southern New England Ultraviolet, Hamden, CT) equipped with 3500 Å lamps. For selected experiments, the affinity-labeled motilin receptor and its relevant fragments were dehydroxylated with endoglycosidase F under the conditions previously reported (22). Membranes were solubilized, and separated by gel electrophoresis using either Laemml conditions with 10% SDS-polyacrylamide gels or 10% NuPAGE gels (InVitrogen, Carlsbad, CA). Labeled products were visualized by autoradiography.

Chemical Cleavage of the Labeled Motilin Receptor—Gel-purified, affinity-labeled native and deglycosylated motilin receptor were digested with cyanogen bromide in 70% formic acid, according to the procedure previously described (22). The products of cleavage were separated on 10% NuPAGE gels using MES running buffer, with labeled products visualized by autoradiography. The apparent molecular masses of radiolabeled receptor fragments were determined by interpolation on a log of the plot of the mobility of Multimark™ protein standards (In-Vitrogen) versus the log values of their apparent masses.

Statistical Analysis—All observations were repeated at least three times in independent experiments and are expressed as means ± S.E. Binding curves were analyzed using the LIGAND program of Munson and Rodbard (23) and were plotted using the nonlinear regression analysis routine for radioligand binding in the Prism software package (GraphPad Software, San Diego, CA).

Results

Binding and Biological Activity Studies with Natural Ligand—A clonal cell line stably expressing motilin receptors was established (CHO-MtIR). The binding of motilin to the human motilin receptors expressed on these cells was specific, saturable, and high affinity, with a K_d of 2.3 ± 0.4 nM motilin (Fig. 2). The calculated receptor density on these cells was 175,000 sites per cell.

Motilin induced a rise in intracellular calcium concentration in CHO-MtIR cells in a concentration-dependent manner, with an EC_{50} of 0.3 ± 0.1 nM motilin (Fig. 2).

Characterization of the Probe—[Bpa^{1},Ile^{13}]motilin contains a photolabile Bpa in the position of Phe as a site for covalent attachment to the receptor and a Tyr residue that is naturally present in position 7 as a site for radiodiodination. An Ile residue was incorporated in the position of Met to eliminate a site for potential oxidative damage during radiolabeling. This motilin analogue was synthesized by manual solid-phase techniques, purified by reversed-phase HPLC, and characterized chemically by mass spectrometry. The t- and d-Bpa stereoisomers of this probe were separated and identified (20), with the t-Bpa being the most active used in the described studies.

The binding of [Bpa^{1},Ile^{13}]motilin to the human motilin receptor was saturable and specific, but displayed a slightly lower affinity (K_d = 12.4 ± 1.0 nM) than natural motilin (Fig. 3). This analogue was a full agonist, inducing a rise in intracellular calcium concentration in CHO-MtIR cells in a concentration-dependent manner with an EC_{50} = 1.5 ± 0.2 nM (Fig. 3).
the peptide competed for binding of 125I-[Ile13]motilin to motilin receptors expressed in membranes from CHO-MtlR cells. Values are calculated as percentages of maximal saturable binding observed in the absence of competitor. They are expressed as means ± S.E. of duplicate data from three independent experiments. Biological activity is shown in the right panel. Increasing concentrations of the peptide increased intracellular calcium concentrations in the CHO-MtlR cells. Values are expressed as the means ± S.E. of at least three independent experiments, with data normalized relative to the maximal response to motilin in each experiment.

**Fig. 2.** Binding and biological activity of motilin. Competition-binding data are shown in the left panel. Increasing concentrations of the peptide competed for binding of 125I-[Ile13]motilin to motilin receptors expressed in membranes from CHO-MtlR cells. Values are calculated as percentages of maximal saturable binding observed in the absence of competitor. They are expressed as means ± S.E. of duplicate data from three independent experiments. Biological activity is shown in the right panel. Increasing concentrations of the peptide increased intracellular calcium concentrations in the CHO-MtlR cells. Values are expressed as the means ± S.E. of at least three independent experiments, with data normalized relative to the maximal response to motilin in each experiment.

**Fig. 3.** Binding and biological activity of [Bpa1,Ile13]motilin. The left panel demonstrates binding analysis. Increasing concentrations of peptide competed for binding of 125I-[Ile13]motilin to membranes from CHO-MtlR cells. Values are calculated as percentages of maximal saturable binding observed in the absence of competitor. They are expressed as means ± S.E. of three independent experiments. Biological activity is shown in the right panel. Increasing concentrations of peptide increased intracellular calcium concentrations in the CHO-MtlR cells. Values are expressed as the means ± S.E. of at least three independent experiments, with data normalized relative to the maximal response to motilin in each experiment.

**Photoaffinity Labeling of the Motilin Receptor—**Membranes prepared from CHO-MtlR were used as source of receptor for affinity labeling. The 125I-[Bpa1,Ile13]motilin analogue covalently saturably labeled two membrane proteins from these cells that migrated on an SDS-polyacrylamide gel at Mr = 78,000 and at Mr = 58,000, with the former representing approximately 80% of labeling (Fig. 4). Photoaffinity labeling of each of these bands was inhibited in a concentration-dependent manner with increasing concentrations of competing unlabeled motilin (IC50 between 1 and 10 nM motilin). Each band had similar apparent affinity for this probe, raising the possibility that they represented distinct glycoforms of a single protein, representing the motilin receptor. Deglycosylation of each of the glycoprotein bands with endoglycosidase F resulted in bands that migrated similarly at approximate Mr = 45,000 (Fig. 5). This corresponded with the expected mass of the core receptor protein. As control, labeled bands of this size were absent in affinity-labeled CHO cell membranes lacking the human motilin receptor.

**Active Site Identification—**Fig. 6 shows the ten fragments that would theoretically be obtained after cyanogen bromide cleavage of the human motilin receptor, ranging in mass from less than 1 to 12 kDa. Conditions for quantitative cleavage of this receptor with cyanogen bromide were established and applied. The spectrum of molecular masses and the presence of glycosylation at distinct sites within the amino terminus and second extracellular loop may, therefore, provide suggestive evidence for the identification of the domain being labeled using this single manipulation.

Cyanogen bromide cleavage of the Mr = 78,000 band covalently labeled by 125I-[Bpa1,Ile13]motilin resulted in two distinct labeled bands (Fig. 6). These had apparent migrations of Mr = 6,000 and Mr = 31,000. The migration of the Mr = 6,000 fragment did not shift further after deglycosylation with endoglycosidase F. Similar treatment of the Mr = 31,000 fragment resulted in its shifting to apparent Mr = 21,000. Considering the mass of the covalently bound ligand probe (2,909 Da) and the glycosylation status of the labeled bands, the most probable identifications of these bands were the cyanogen bromide fragments that included the first and second extracellular loop domains.

To further establish these identities, two motilin receptor mutants were constructed that introduced additional sites of cyanogen bromide cleavage at new Met residues within each of these candidate domains. These included L98M and I185M...
motilin receptor constructs. Fig. 7 shows that motilin bound with normal affinity (Ki values of 3.4 ± 1.1 nM motilin at the L98M receptor and 7.0 ± 0.2 nM motilin at the I185M receptor) and stimulated a normal intracellular calcium concentration response in both of these receptor constructs (EC50 values of 1.8 ± 0.6 nM motilin at the L98M receptor and 3.7 ± 0.8 nM motilin at the I185M receptor).

The 125I-[Bpa1,Ile13]motilin probe efficiently and saturably labeled the wild type and mutant motilin receptor constructs expressed on the COS cells (Fig. 8). Glycosylation of the motilin receptor was different in the COS cells from that in the CHO cell line, with the labeled glycosylated cyanogen bromide fragment in the COS cells migrating as a broad band between Mr = 42,000–52,000 rather than at Mr = 31,000 (observed in the CHO cell line). Each of the non-glycosylated fragments from both cell types migrated similarly. Fig. 8 shows the effects of cyanogen bromide cleavage of the labeled receptor constructs. As predicted, the labeled Mr = 6,000 band from the wild type receptor shifted to approximately Mr = 5,000 in the L98M receptor. Also as expected, the labeled glycosylated Mr = 42,000 band from the wild type receptor shifted to a non-glycosylated band migrating at Mr = 12,000 in the I185M receptor (although the cleavage was not fully complete). This confirmed that the second region of labeling was between residues 130 and 185 of the receptor.

**DISCUSSION**

The G protein-coupled receptor superfamily is remarkable for the diversity of structures of its natural agonist ligands, ranging from extremely small photons, odorants, and biogenic
amines to larger peptides, glycoproteins, and even viral particles. It is well recognized that these receptors can be clustered based on sequence homology, with structurally related groups often representing targets of structurally related ligands (24). It has been assumed that themes of ligand binding are analogous in closely related receptors within this superfamily.

There is substantial structural homology between the motilin receptor and the previously cloned growth hormone secretagogue receptors (8–10). This is most marked in the predicted transmembrane domains, where these receptors share 86% sequence identity. With the recent identification of natural ligands for one of the growth hormone secretagogue receptors (a 28-amino acid peptide, ghrelin, and its splice variant representing des-Gln14-ghrelin) (25, 26), a structural relationship between these ligands and motilin also became clear. Alignment of motilin with ghrelin revealed that 8 of its 22 amino acid residues were identical (motilin residues Phe5, Glu9, Gln11, Arg12, Gln14, Lys16, Glu17, and Lys20). Of interest, these were distributed throughout motilin, with several of these residues present within the amino-terminal pharmacophoric domain of this hormone.

In this work, we have established a CHO-MtIR cell line that overexpresses the fully functional recombinant human motilin receptor, and we have developed a radioiodinatable analogue of motilin that incorporates a photolabile residue within the pharmacophoric domain, in position 1 of this peptide. This probe represents a full agonist that binds to the motilin receptor with high affinity in the presence of several of these residues within the amino-terminal pharmacophoric domain of this hormone. It efficiently labeled two cyanogen bromide fragments of the receptor that include the first and second extracellular loop domains. This is consistent with the pharmacophoric domain of motilin binding in close proximity to these regions of the receptor. These data provide the first direct evidence for molecular approximations between a residue within a motilin-like agonist and this receptor, and begin the definition of important domains within this receptor.

It is of particular interest that alignment of the motilin receptor with the growth hormone secretagogue receptors reveals that this large second extracellular loop domain that is covalently labeled in this study is absent in the growth hormone secretagogue receptors. There is a 40-residue insertion in the motilin receptor relative to those receptors in this region. An analogous region can, therefore, not be involved in the binding of ghrelin ligands to those receptors.

It is noteworthy that ghrelin and des-Gln14-ghrelin both have a unique post-translational modification, representing N-octanoylation of Ser3, that has been shown to be necessary for their biological activity, the pulsatile secretion of growth hormone (25, 26). No such modification has been described for motilin. It is quite possible that this biologically essential fatty acid acylation of the natural ligands for the growth hormone secretagogue receptors establishes a distinct mode of binding from that observed for the receptor that normally interacts with non-acylated motilin. The incorporation of a modification that is so hydrophobic may bring these ligands closer to the lipid bilayer for their sites of recognition than for a non-modified peptide ligand.

The active sites of G protein-coupled receptors are of substantial interest for rational drug design. The identification of the ligand-binding domain of the human motilin receptor may lead to the development of more selective and potent agonists acting at this important receptor. Particularly in view of the well described gastrointestinal prokinetic properties of motilin and of the established clinical efficacy for the non-peptidyl motilin receptor ligand, erythromycin (4), new compounds may be of potential benefit to patients with diabetic gastroparesis (3) or other disturbances of normal food propulsion.

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