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Demonstration of a specific site of covalent labeling of the human motilin receptor using a biologically active photolabile motilin analogue

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Running Title: Ligand-binding domain of the motilin receptor

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ABBREVIATIONS: Bpa, benzoyl-phenylalanine; CHO, Chinese hamster ovary; CHO-MtlR, Chinese hamster ovary cell lines stably expressing the wild-type human motilin receptor; CNBr, cyanogen bromide; Endo F, endoglycosidase F; G protein, guanine nucleotide-binding protein; Iodo-beads, N-chlorobenzenesulfonamide; KRH, Krebs-Ringer/HEPES; Lys-C, Endoproteinase Lys-C; Fura-2-AM, Fura-2 acetoxymethyl ester; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

ABSTRACT

The motilin receptor belongs to a group of Class I G protein-coupled receptors that also includes growth hormone secretagogue and ghrelin receptors. These represent clinically useful targets for pharmacotherapy. Their potentially unique structures and the molecular basis of their binding are not yet clear. We previously reported the initial affinity labeling of a region within this receptor (cyanogen bromide fragment extending from the first to the second extracellular loop) using a position one photolabile motilin analogue. To extend our understanding of the molecular basis of motilin binding, we have developed an additional radioiodinatable motilin analogue probe having site of covalent attachment in position five. This was a full agonist that bound to the motilin receptor specifically and with high affinity, and that efficiently established a single covalent bond to its receptor. Sequential chemical and enzymatic cleavage of labeled wild type and mutant motilin receptor constructs established that the region of labeling was within the third extracellular loop. This was further localized to Phe^{332} using radiochemical Edman degradation sequencing. These data provide the first spatial approximation constraint that can be utilized in the docking of this peptide ligand to its receptor. We hope that a series of such constraints can be determined to provide adequate structural information to begin to elucidate the conformation of this agonist-bound receptor, and to ultimately be useful in the rational design of drugs acting at this important target.

The detailed understanding of the basis for binding a ligand by a receptor can help refine our understanding of the conformation of the receptor and of the molecular basis for its activation. While all members of the superfamily of guanine nucleotide-binding protein (G protein)-coupled receptors have structural similarities, with seven hydrophobic segments that are believed to traverse the plasma membrane, the details of even this most fundamental characteristic varies with the family of these receptors (Kolakowski, 1994; Ji, et al., 1998). This superfamily is also remarkable for the structural diversity of the natural ligands that activate various members, with themes ranging from small photons and biogenic amines that bind within the confluence of transmembrane segments to larger peptide and glycoprotein ligands that bind to extracellular tail and loop domains.

The class I G protein-coupled receptors that are activated by motilin and ghrelin were recognized only recently (Feighner, et al., 1999; Howard, et al., 1996; McKee, et al., 1997), and cluster together in sequence analysis, having substantial structural similarity. This has typically been a predictor of similarity in ligand structure and mechanism of binding and activation (Kolakowski, 1994). While these natural ligands have extensive sequence homology, ghrelin requires a unique post-translational modification for its activity that is not present or required for motilin (Kojima, et al., 1999). This represents the N-octanoylation of the Ser residue in its third position. 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 predominant carboxyl-terminal,

sequence determinants for its selectivity of binding and action (Peeters, et al., 1992; Poitras, et al., 1992; Poitras, et al., 1994; Miller, et al., 1995; Boulanger, et al., 1995). This raises the possibility that the mechanisms of ligand binding and activation of these receptors might be distinct.

We have recently performed the initial photoaffinity labeling of the motilin receptor, using a probe with photolabile site of covalent attachment in position one of the peptide (Coulie, et al., 2001). That probe labeled a region of this receptor (a cyanogen bromide fragment) that included the predicted second intracellular loop domain, the fourth transmembrane segment, and the second extracellular loop (Coulie, et al., 2001). This was potentially quite interesting, since this extracellular loop domain was quite long in the motilin receptor, including a 67 residue insertion that was not present in the otherwise structurally-similar receptor activated by ghrelin (Howard, et al., 1996). We recently performed extensive mutagenesis on this loop domain and were surprised to find that the entire insertion domain could be deleted without having negative impact on motilin binding or biological activity (Matsuura, et al., 2002). The regions of this loop that were functionally important in that series were at both ends of the loop, adjacent to the plasma membrane.

In the current study, we extend our understanding of the molecular basis of motilin binding by developing a second photolabile agonist probe and using it to localize the first residue-residue approximation constraint for this ligand-receptor pair. This probe incorporates a site of covalent

attachment in position five of a motilin analogue, within the pharmacophoric domain of this peptide (Peeters, et al., 1992; Poitras, et al., 1992; Poitras, et al., 1994; Miller, et al., 1995; Boulanger, et al., 1995). We utilized a series of specific cleavage reactions and a new receptor mutant to localize the region of labeling to a region within the third extracellular loop domain. This was further refined to demonstrate labeling of Phe^{332}. Thus, it appears that, like several other peptide ligands (Ji, et al., 1998), motilin binds to extracellular loop domains of this receptor. This type of constraint will be quite useful in the initial docking of this peptide to its receptor as meaningful conformational models become available.

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Materials and Methods

Materials. The solid phase oxidant, N-chlorobenzenesulfonamide (Iodo-beads), cyanogen bromide (CNBr), and 2-(2'-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine (skatole) were from Pierce Chemical Co. (Rockford, IL). Fura-2 acetoxymethyl ester (Fura-2-AM) was from Molecular Probes (Eugene, OR). Endopeptidase Lys-C (Lys-C) was from Roche Molecular Biochemicals (Indianapolis, IN). Endoglycosidase F (Endo F) was prepared in our laboratory, as we previously reported (Pearson, et al., 1987). All other reagents were analytical grade.

Peptides. The forms of motilin that were utilized corresponded to the human sequence with the noted changes. We synthesized natural human motilin, [Ile^{13}]motilin, and [Bpa^{5},Ile^{13}]motilin using manual solid-phase techniques (Powers, et al., 1988a; Coulie, et al., 2001). The latter probe was designed to incorporate a photolabile benzoyl-phenylalanine (Bpa) in the position of Phe^{5} as a site for covalent labeling of the receptor and an Ile residue in the position of Met^{13} to eliminate a site for potential oxidative damage during radiolabeling. This probe contained a Tyr residue that is naturally present in position 7 as a site for radioiodination. These peptides were purified to homogeneity by sequential steps of reversed-phase high performance liquid chromatography (Powers, et al., 1988b). Peptides incorporating the D- and L-Bpa stereoisomers were separated and identified using the technique of Miller and Kaiser (Miller and Kaiser, 1988). The L-Bpa peptide was used in the present studies. The chemical identities of these peptides were established by mass spectrometry.

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[Ile^{13}]motilin and [Bpa^{5},Ile^{13}]motilin were radioiodinated on residue Tyr^{7} using exposure to the solid-phase oxidant, Iodo-beads, as previously described (Powers, et al., 1988b). They were purified by reversed-phase high performance liquid chromatography to yield specific radioactivities of 2,000 Ci/mmol (Powers, et al., 1988b).

Receptor preparations. The human motilin receptor cDNA was kindly provided by S.D. Feighner and A.D. Howard of Merck Research Laboratories, Rahway, NJ (Feighner, et al., 1999). Mutant motilin receptor constructs were prepared using an oligonucleotide-directed approach with the QuikChange^{TM} Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). Development of two new motilin receptor constructs was necessary for this study. This included a mutant to help localize the domain that was affinity labeled by replacing motilin receptor residue Gln^{334} predicted to be within the seventh transmembrane segment with Met (Q334M) to incorporate an additional site for CNBr cleavage. It also included a mutant that incorporated two Cys residues in the intracellular carboxyl-terminal tail domain (replacement of Lys^{368} and Lys^{374} with Cys residues, K368,374C) for covalent coupling of the affinity labeled receptor fragment to solid-phase glass beads for radiochemical sequencing. Sequences of these constructs were confirmed by direct DNA sequencing.

COS-1 and Chinese hamster ovary (CHO-K1) cells (American Type Culture Collection,

Manassas, VA) that do not naturally express the motilin receptor were used as cellular background for expression of various motilin receptor constructs. We previously established and characterized a CHO cell line stably expressing the wild type human motilin receptor (CHO-MtlR) (Coulie, et al., 2001). For this work, we also established an additional CHO cell line that stably expressed the dual Cys mutant motilin receptor (K368,374C) using similar procedures (Coulie, et al., 2001). 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). They were passaged approximately twice a week and were lifted mechanically before use.

The Q334M motilin receptor construct was expressed transiently in COS cells after transfection using a modified DEAE-dextran protocol (Lopata, et al., 1984). Cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 5% Fetal Clone-2. For receptor binding studies, cells were lifted mechanically three days after transfection, and were used to prepare enriched plasma membranes (Hadac, et al., 1996). For biological activity studies, cells were studied in an appropriate culture dish three days after transfection.

Receptor binding assay. Plasma membranes were prepared from receptor-bearing cells using sonication and sucrose gradient centrifugation, as we previously reported (Hadac, et al., 1996). For binding characterization, enriched membranes (5-10 μg protein) were incubated with a constant

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amount of radioligand, ^{125}I-[Ile^{13}]motilin (3-5 pM), in the presence of increasing concentrations of unlabeled motilin or [Bpa^{5},Ile^{13}]motilin (ranging from 0 to 1 μM). Incubations were carried out for 1 hr at room temperature in Krebs-Ringers-HEPES (KRH) medium containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 0.01% soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.2% bovine serum albumin. Bound and free radioligand were separated using a Skatron cell harvester (Molecular Devices, Sunnyvale, CA) with receptor-binding filtermats, and bound radioactivity was quantified in a γ-spectrometer. The intact cell binding assay was performed in 24-well tissue culture plates. Non-specific binding was determined in the presence of 1 μM motilin and represented less than 20% of total radioligand bound. All assays were performed in duplicate and were repeated at least three times in independent experiments.

Biological activity characterization. The agonist activity of [Bpa^{5},Ile^{13}]motilin was studied using an assay for stimulation of intracellular calcium concentration in cells expressing motilin receptor constructs (Grynkiewicz, et al., 1985). Natural human motilin was used as a positive control. In this assay, 2 x 10^{6} receptor-bearing cells were loaded with 5 μM Fura-2-AM (Molecular Probes) in calcium-free KRH medium for 20 min at 37°C. After washing, cells were stimulated with varying concentrations of peptide ligand at 37°C. Fluorescence was quantified in a Perkin-Elmer LS50B luminescence spectrometer (Norwalk, CT). Excitation was performed at 340 and 380 nm, and emission was quantified at 520 nm, with calcium concentration calculated from the ratio, as described by

Grykiewicz et al (Grynkiewicz, et al., 1985). The peak intracellular calcium transient was utilized to determine the agonist concentration-dependence of this biological response. Basal levels of calcium were determined by calculating the intracellular calcium concentration in the absence of agonist stimulation, whereas maximal levels were determined as the peak intracellular calcium concentration achieved in the presence of 1 μM agonist. Stimulated values of intracellular calcium were determined by subtraction of the basal levels from the maximal levels of intracellular calcium concentration in a given set of experiments. All assays were performed in duplicate and were repeated at least three times in independent experiments.

Photoaffinity labeling of the motilin receptor. For this, enriched plasma membranes (100 μg) were incubated for 1 hr in the dark at 25°C with ^{125}I-[Bpa^{5},Ile^{13}]motilin (100 pM) in the absence or presence of increasing concentrations of non-radiolabeled motilin. 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 3,500 Å lamps. Membranes were then washed, solubilized and separated by gel electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) using conditions described by Laemmli (Laemmli, 1970). Labeled products were visualized by autoradiography. For selected experiments, the affinity-labeled motilin receptor and its relevant fragments were deglycosylated with endoglycosidase F under the conditions we previously described (Hadac, et al., 1998).

Chemical and enzymatic cleavage of the labeled motilin receptor. Gel-purified, affinity-labeled native and deglycosylated motilin receptors were digested separately or sequentially with CNBr and/or endopeptidase Lys-C, as we previously described (Dong, et al., 1999). Further cleavage of the CNBr cleaved fragment was performed with 2 mg/ml skatole in 70 % (vol/vol) acetic acid, according to the method previously reported (Dong, et al., 1999). The products of cleavage were separated on 10% NuPAGE gels (Invitrogen, Carlsbad, CA) using MES running buffer, with labeled products visualized by autoradiography. The apparent molecular masses of radiolabeled receptor fragments were determined by interpolation on a plot of the mobility of Multimark™ protein standards (Invitrogen) versus the log values of their apparent masses.

Identification of site of covalent attachment. After achieving definitive identification of the receptor fragment that was labeled with [Bpa^{5},Ile^{13}]motilin, its site of attachment was determined using radiochemical Edman degradation sequencing, as we previously described (Dong, et al., 1999). This procedure involved in the use of cross-linking through the Cys residues within a protein fragment to N-(2-aminoethyl-1)-3-aminopropyl glass beads (Sigma). For this, we utilized the K368,374C receptor construct expressed on a CHO cell line. This mutant receptor was affinity labeled with [Bpa^{5},Ile^{13}]motilin and cleaved by CNBr. The gel-purified radiolabeled cleavage product was covalently coupled through its Cys residues to maleimidobenzoyl succinimide-activated

N-(2-aminoethyl-1)-3-aminopropyl glass beads. This was followed by repetitive cycles of manual Edman degradation with quantitation of radioactivity released in each cycle. This procedure was performed at least three times in independent experiments.

Statistical analysis. All observations are expressed as means±S.E.M. Binding curves were analyzed using the Ligand program of Munson and Rodbard (Munson and Rodbard, 1980) and plotted using the non-linear regression analysis in the Prism software package (GraphPad Software, San Diego, CA).

Results

Characterization of the probe. [Bpa^{5},Ile^{13}]motilin was synthesized and purified to homogeneity. It was characterized both chemically by mass spectrometry and functionally in binding and biological activity assays. This analogue bound to the motilin receptor saturably and specifically, but displayed a lower affinity (K_i=50±7 nM) than natural motilin (K_i=2.3±0.4 nM) (Fig 1). The binding of the peptide that incorporated the D-Bpa residue displayed a much lower affinity (K_i=420±67 nM) than the L-Bpa peptide (K_i=50±7 nM), and was therefore not considered for further use. The lower affinity of the [D-Bpa^{5},Ile^{13}]motilin supports the previous interpretation that the fifth residue of motilin is involved in stabilizing the bioactive conformation of the peptide (Peeters, et al., 1992).

[Bpa^{5},Ile^{13}]motilin represented a fully efficacious agonist acting at the motilin receptor (Fig 1). It stimulated an increase in intracellular calcium concentrations in receptor-bearing CHO-MtlR cells in a concentration-dependent manner (EC_{50}=4.3±0.9 nM), although its potency was lower than that of natural motilin (EC_{50}=0.3±0.1 nM).

Photoaffinity labeling of the motilin receptor. Like the [Bpa^{1},Ile^{13}]motilin probe that we previously reported (Coulie, et al., 2001), the [Bpa^{5},Ile^{13}]motilin analogue covalently labeled two membrane proteins from receptor-bearing CHO-MtlR cells. The major band migrated on a 10% SDS-polyacrylamide gel at apparent M_r=78,000, with the minor band at M_r=58,000 (Fig 2).

Photoaffinity labeling of each of these bands was inhibited in parallel in a concentration-dependent manner with increasing concentrations of unlabeled motilin. Like the earlier study (Coulie, et al., 2001), both bands represented distinct glycoforms of motilin receptor, since deglycosylation of each of the bands with endoglycosidase F resulted in bands that migrated similarly at approximate M_r=45,000, corresponding with the expected mass of the core receptor protein (Fig 2). Bands of this size were absent in samples prepared from non-receptor bearing parental CHO-K1 cell membranes (data not shown). Figure 2 also includes the densitometric analysis of the labeling of the M_r=78,000 band in three similar experiments. The IC_{50} value for this competition was between 1 and 10 nM.

Identification of the domain of labeling. CNBr cleavage was used to gain initial insight into the domain of labeling of the motilin receptor with the [Bpa^5,Ile^{13}]motilin probe. CNBr cleavage of the motilin receptor would theoretically result in 10 fragments ranging in molecular weight from less than 1 to 12.5 kD, with two fragments containing glycosylation sites (Fig 3). As shown in Figure 3, CNBr cleavage of the intact labeled motilin receptor yielded a band migrating at approximate M_r=14,000 that did not shift after deglycosylation with endoglycosidase F. Considering the mass of the covalently bound ligand probe (2,677 Da) and the non-glycosylated nature of the labeled fragment, two candidate fragments best fit these data. The first candidate represents the fragment spanning the fifth transmembrane segment, the third intracellular loop, the sixth transmembrane segment, and the third extracellular loop (fragment 7). The other candidate represents the fragment incorporating the seventh

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transmembrane segment, with extracellular and intracellular domains on either side (fragment 9).

Interestingly, the characteristics of these two fragments are quite distinct. Fragment 7 contains three tryptophan residues (Trp^{250}, Trp^{275}, and Trp^{311}) that can be cleaved by skatole and no lysine residues. In contrast, fragment 9 contains four lysine residues (Lys^{360}, Lys^{361}, Lys^{368}, and Lys^{374}) that can be cleaved by endopeptidase Lys-C, and no tryptophan residues. Therefore, further cleavage reactions of the labeled CNBr fragment with Lys-C and skatole were performed to identify which of the two fragments represented the best candidate to contain the site of labeling with the [Bpa^{5},Ile^{13}]motilin probe. As shown in Figure 3, cleavage of the labeled M_r=14,000 CNBr fragment with skatole did not yield a visible shift, while cleavage with Lys-C shifted the band to M_r=5,000. It should be noted that the probe would also be cleaved by Lys-C, resulting in a truncation of 6 residues at the carboxyl terminus (~0.7 kD), which would be expected to yield only a small or non-observable shift in migration. Taken together, these data indicated that the fragment 9 was the best candidate to contain the site of labeling with the [Bpa^{5},Ile^{13}]motilin probe, likely within the region Tyr^{331}-Lys^{360}.

To further establish this identity and localize the site of labeling, a receptor mutant was constructed with Gln^{334} changed to Met (Q334M) to introduce an additional site for CNBr cleavage within the expected fragment. This was expressed transiently in COS cells. Figure 4 shows that this construct bound motilin with normal affinity (K_i=0.85±0.19 nM) and was able to initiate normal

signaling (EC_{50} for motilin stimulation of an intracellular calcium response of 8.4±4.9 nM). The [Bpa^{5},Ile^{13}]motilin probe efficiently and saturably affinity labeled the Q334M motilin receptor construct expressed in COS cells (Fig 4). Shown also is its labeling of the wild type motilin receptor expressed in the same cells, since the glycosylation of the motilin receptor on these cells was different from that observed in the CHO-MtlR cells (Coulie, et al., 2001). As expected, the labeled M_r=14,000 CNBr fragment from the wild type receptor shifted to an approximate M_r=4,000 band in the Q334M receptor construct (Fig 4). These results definitively establish the identity of the labeled CNBr fragment of the motilin receptor, while, at the same time, helping to refine the labeled domain. The domain labeled includes the region between receptor residues Tyr^{331} and Gln^{334}.

Identification of the specific site of labeling. For these studies, a CHO cell line was generated that stably expressed a receptor mutant with residues Lys^{368} and Lys^{374} both changed to Cys (K368,374C) to introduce residues that would allow it to be covalently coupled via their sulfhydryl groups to glass beads for sequencing. Figure 5 shows that motilin bound with normal affinity (K_i=0.6±0.1 nM) and stimulated a normal intracellular calcium concentration response (EC_{50}=0.3±0.1 nM) in these cells. The [Bpa^{5},Ile^{13}]motilin probe efficiently and saturably labeled the K368,374C construct (Fig 5). As expected, CNBr cleavage of this construct yielded a labeled band that migrated at the same position as the M_r=14,000 CNBr fragment from the labeled wild type receptor expressed in CHO-MtlR cells.

Identification of the specific residue that was labeled with the [Bpa^{5},Ile^{13}]motilin probe was achieved by manual Edman degradation sequencing of the purified labeled CNBr fragment of the K368,374C construct attached to the glass beads (Fig 6). This construct was engineered to allow covalent attachment to the beads in a location that would not interfere with the sequencing reactions. In three independent experiments, the peak in eluted radioactivity consistently appeared in cycle 2, corresponding to the covalent labeling of receptor residue Phe^{332}.

Discussion

In this series of studies, we have demonstrated the spatial approximation between a residue within a full agonist analogue of motilin as this ligand is normally docked to its receptor and a distinct residue within the receptor molecule. While previous photoaffinity labeling and receptor mutagenesis studies have provided insights into broad regions of this receptor that are adjacent to bound hormone and that are functionally important for motilin binding and action, this constraint provided by the spatial approximation between a photolabile residue intrinsic to the pharmacophoric domain of motilin and a distinct residue within its receptor provides the first insights that can contribute toward meaningful modeling of the binding of motilin-like peptidyl ligands to this important receptor.

Motilin is a physiologically important linear 22-residue peptide that stimulates gastrointestinal motility (Janssens, et al., 1983; Itoh, 1997). It reaches peak levels in the circulation cyclically through the day, correlating with an increase in interdigestive motor activity in the stomach and small intestine. The prokinetic effect of this hormone is believed to play an important role in the normal housekeeping of the gut, purging any residual luminal material in preparation for the subsequent meal. It also represents a potentially important pharmacological agent to stimulate gastric emptying and intestinal transit in clinical dysmotility states (Janssens, et al., 1983; Itoh, 1997). Indeed, this represents the rationale for the therapeutic use of erythromycin, found to represent a weak agonist acting at the motilin receptor (Janssens, et al., 1990; Peeters, 1993).

The current work represents part of an experimental strategy to gain insights into the structure of the motilin receptor and its mode of agonist ligand binding, in an effort to ultimately develop the next generation of drugs that can act at the motilin receptor. Little is currently known of the mode of binding of this peptide to its receptor. There is reason to believe that the peptide ligands for the receptors in the motilin receptor family will bind to their receptors in a manner different from the natural peptide ligands for other Class I G protein-coupled receptors. This relates to the finite carboxyl-terminal pharmacophoric domain typical of the other peptides that bind to Class I G protein-coupled receptors (Kolakowski, 1994), while motilin has its major region of selectivity within its amino-terminal decapeptide (Poitras, et al., 1992; Feighner, et al., 1999). The unique structural characteristics of the natural ligand for another closely related receptor in the motilin receptor family, the ghrelin receptor having a ligand with a functionally-critical N-octanoyl moiety at the serine residue in the third position, add to the interest in how the natural ligands for the motilin receptor family might dock with their receptors.

The most extensive literature contributing to our understanding of mechanisms of ligand binding to a receptor come from structure-activity studies, including receptor mutagenesis. To date, the only published mutagenesis data for any receptor in the motilin receptor family is limited to a detailed analysis of the predicted very large second extracellular loop region of the motilin receptor (Matsuura,

et al., 2002). An extensive series of deletion and replacement mutations throughout that region revealed that, in addition to the cysteine residue in this region (Cys^{235}) that is likely involved in the heavily conserved disulfide bond linking the first and second loops of most G protein-coupled receptors, alanine-replacement mutagenesis of only a few of the residues that are predicted to be at the membranous interface (Val^{179}, Leu^{245}, Arg^{246}) had any negative functional impact on natural motilin binding or biological activity. Of interest, the same mutations had no negative effect on biological responses to the non-peptidyl agonist, erythromycin. The functional role of this large second extracellular loop of the motilin receptor is, therefore, currently indeterminate. Other regions of the motilin receptor have not yet been systematically analyzed by this methodology.

Photoaffinity data, such as that provided by the current studies, can contribute direct insights into spatial approximation between distinct residues within a docked ligand and within its receptor. However, prior to this, such studies have been limited to the affinity labeling of the entire intact receptor or of large regions within this receptor (Coulie, et al., 2001). Previous use of a probe with site of covalent attachment in position one labeled a region of this receptor (a cyanogen bromide fragment) that included the predicted second intracellular loop domain, the fourth transmembrane segment, and the second extracellular loop (Coulie, et al., 2001). The current data, using a position five probe, established spatial approximation with a distinct receptor residue within the third extracellular loop region. This supports the general theme of other members of this superfamily in which large peptides

having diffuse pharmacophoric regions bind to extracellular epitopes of the receptor. The generation of residue-residue approximation constraints will have to be replicated for additional loci throughout the pharmacophoric region of motilin to contribute to the meaningful docking of this ligand to its receptor.

As data from both receptor mutagenesis studies and photoaffinity studies accumulate, a more complete picture of critical regions and sites of spatial approximation between ligands and this receptor will emerge. These can contribute to not only effective ligand docking, but also to refinement of our understanding of the conformation of the receptor itself. This will become effective once such sets of data are comprehensive and representative of the entire ligand pharmacophore and the full surface of the receptor.

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Footnotes

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Figure Legends

Fig. 1. Functional characterization of the [Bpa^{5},Ile^{13}]motilin probe. Shown in the left panel is the competition-binding curve reflecting the ability of increasing concentrations of unlabeled peptide to displace the binding of ^{125}I-[Ile^{13}]motilin to membranes from CHO-MtlR cells. Values represent percentages of maximal saturable binding that were observed in the absence of competitor. They are expressed as means±S.E.M. of duplicate data from three independent experiments. Shown in the right panel are intracellular calcium responses to increasing concentrations of [Bpa^{5},Ile^{13}]motilin in CHO-MtlR cells. The basal level of intracellular calcium was 138 ± 20 nM, with the maximal-stimulated level reaching 198 ± 32 nM. Values are expressed as means±S.E.M. of data from three independent experiments, with data normalized relative to the maximal response to motilin.

Fig. 2. Photoaffinity labeling of the motilin receptor. Shown is a representative autograph of a 10% SDS-PAGE gel used to separate the products of photoaffinity labeling of the motilin receptor with ^{125}I-[Bpa^{5},Ile^{13}]motilin in the absence and presence of increasing concentrations of unlabeled motilin (upper panel). Glycoforms of the affinity labeled receptor migrated at approximate M_r=78,000 and M_r=58,000. After deglycosylation with Endo F, both bands shifted to apparent M_r=45,000 (bottom panel). Shown in the middle panel is the densitometric analysis of labeling of the major band in three similar experiments, with data points expressed as means±S.E.M.

Fig. 3. Chemical and enzymatic cleavage of the affinity labeled motilin receptor. Shown in the left panel is a diagram of the predicted sites of CNBr cleavage of the human motilin receptor, along with the masses of protein cores of these fragments. Shown in the middle panel is a typical autoradiograph of a 10% NuPAGE gel used to separate the products of CNBr cleavage of the motilin receptor that had been labeled with ^{125}I-[Bpa^{1},Ile^{13}]motilin. Cleavage of the native receptor yielded a band migrating at M_r=14,000 that was not affected by deglycosylation with Endo F. Fragment 7 and 9 were the best candidates to fit these data. The right panel reflects the migration of the products of further cleavage of this band using endoproteinase Lys-C and skatole. The band shifted to M_r=5,000 after Lys-C cleavage, but was not affected by skatole cleavage. These data suggest that the fragment 9 contained the site of labeling, likely within the region Tyr^{331}-Lys^{360}.

Fig. 4. Further localization of the ligand binding domain by CNBr cleavage of the affinity labeled Q334M motilin receptor mutant. Shown in the upper panel are motilin competition-binding data and intracellular calcium concentration response data for the Q334M motilin receptor construct expressed in COS cells. The basal level of intracellular calcium was 140 ± 18 nM, with the maximal stimulated level reaching 209 ± 28 nM. Values are illustrated as in Figure 1, representing means±S.E.M. of data from three independent experiments performed in duplicate. Shown in the bottom panel are the saturable affinity labeling of the Q334M construct and the wild type motilin receptor and the differential migration of glycoforms in COS and CHO cells. Shown also in the bottom panel is an autoradiograph of

a 10% NuPAGE gel used to separate the products of CNBr cleavage of the affinity labeled Q334M construct. As in CHO cells, the CNBr cleavage of the wild type receptor expressed in COS cells yielded a band migrating at approximate M_r=14,000. This band shifted to M_r=4,000 in the Q334M motilin receptor, indicating the site of labeling being within the region Tyr^{331} and Gln^{334}.

Fig. 5. Characterization of the K368,374C construct. Shown in the upper panel are motilin competition-binding data and intracellular calcium concentration response data for the K368,374C construct stably expressed in CHO cells. The basal level of intracellular calcium was 145 ± 27 nM, with the maximal stimulated level reaching 210 ± 30 nM. Values are illustrated as in Figure 1, with means±S.E.M. of data from three independent experiments performed in duplicate. Bottom panel shows the saturable affinity labeling of the K368,374C construct, along with its CNBr cleavage to yield a band migrating at M_r=14,000, similar to that of the CNBr fragment from the wild type motilin receptor.

Fig. 6. Edman degradation sequencing of the fragment labeled with [Bpa^5,Ile^{13}]motilin probe. Shown is the profile of elution of radioactivity from Edman degradation sequencing of the purified CNBr fragment (Tyr^{331}-Met^{411}) of the K368,374C motilin receptor mutant labeled with ^{125}I-[Bpa^5,Ile^{13}]motilin. The peak in eluted radioactivity was consistently observed in cycle 2 in three independent experiments. This corresponds to covalent attachment of Bpa^5 of the probe to Phe^{332} of the receptor.

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Figure 1

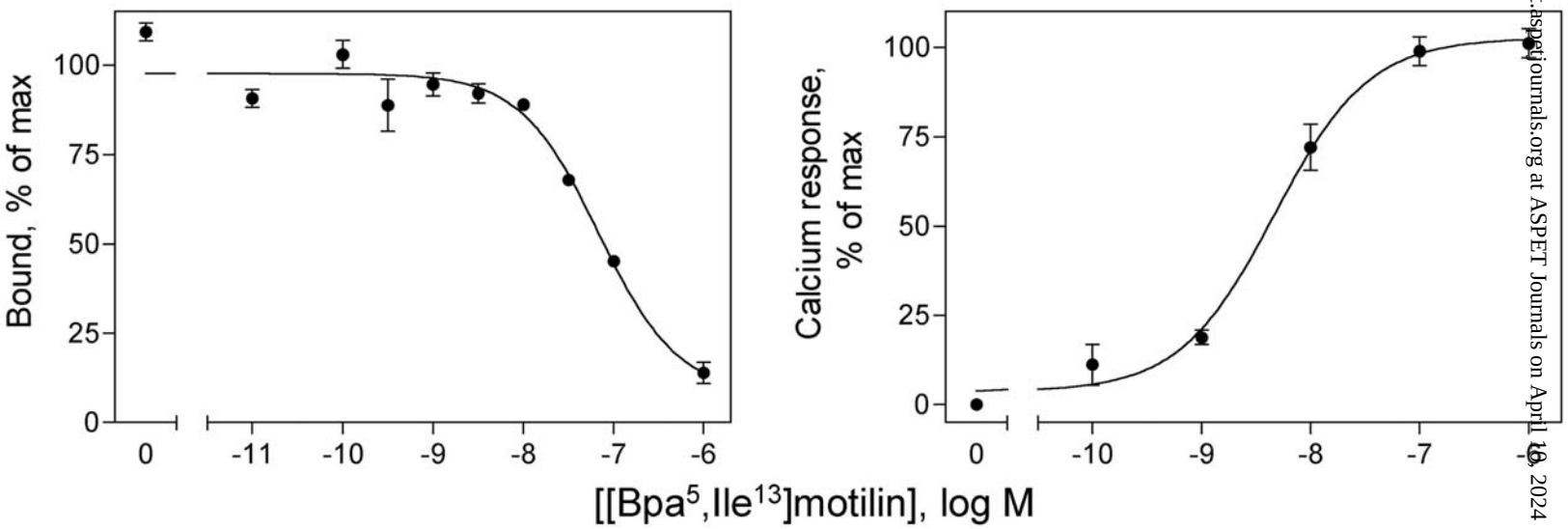

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Figure 2

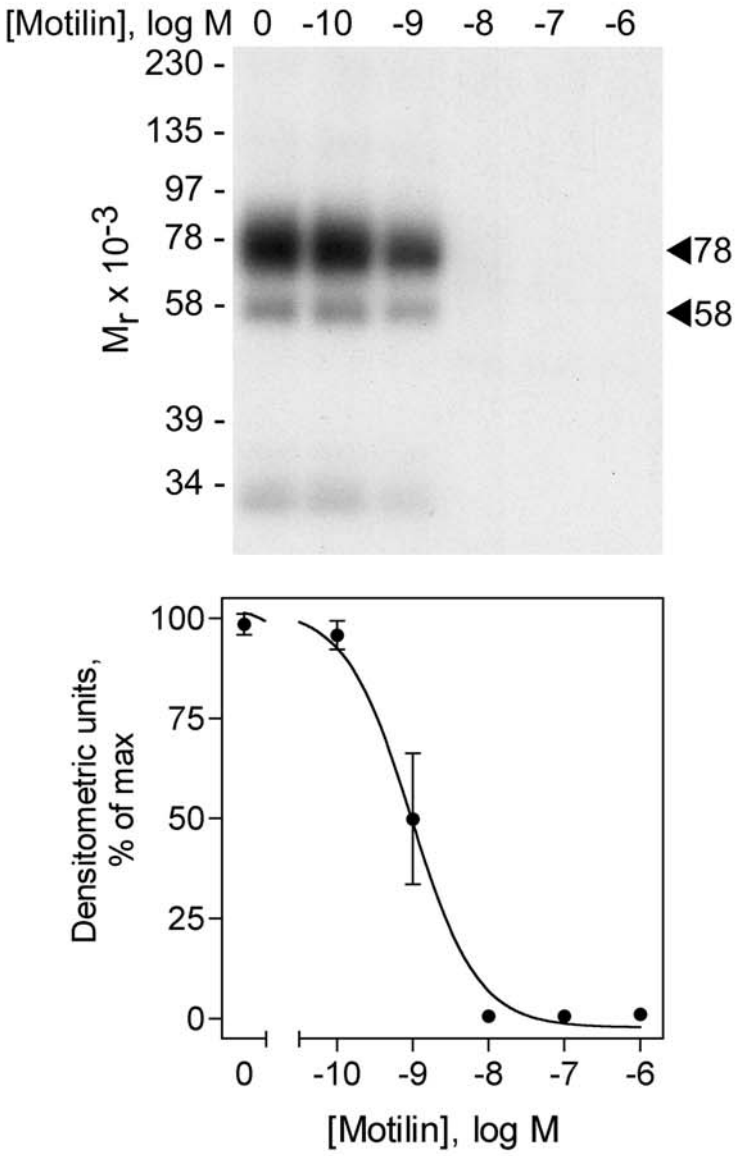

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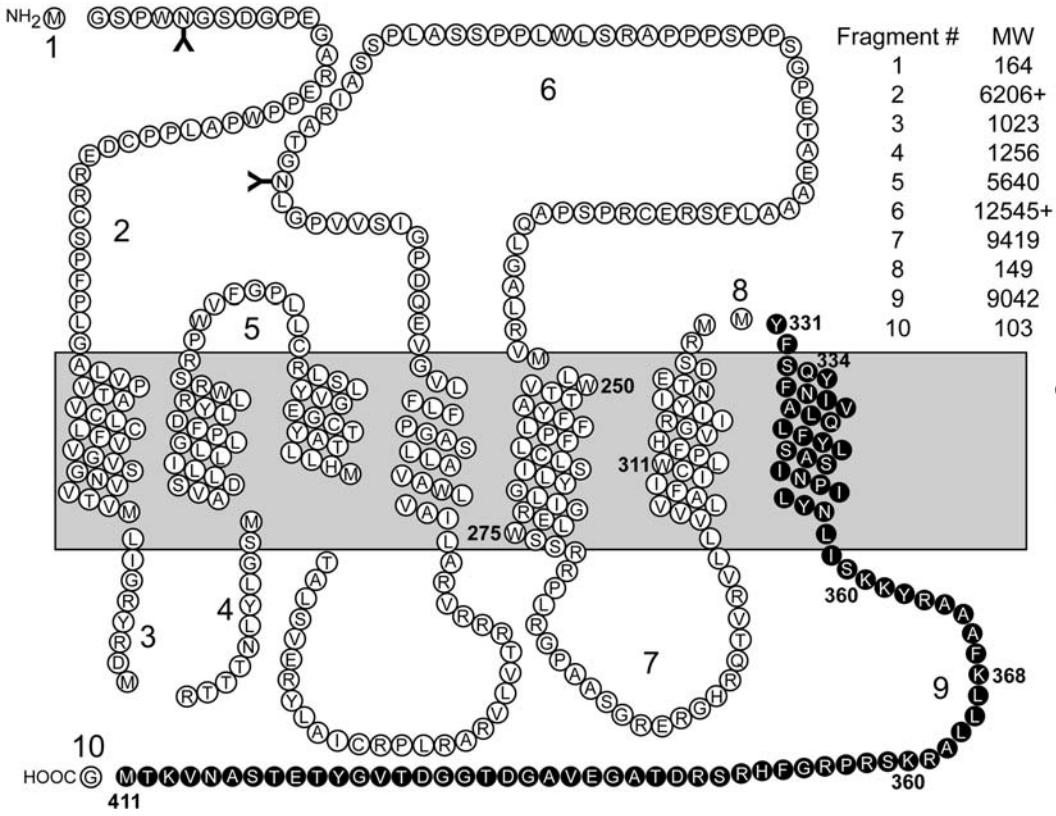
Figure 3



Fragment #	MW
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2	6206+
3	1023
4	1256
5	5640
6	12545+
7	9419
8	149
9	9042
10	103

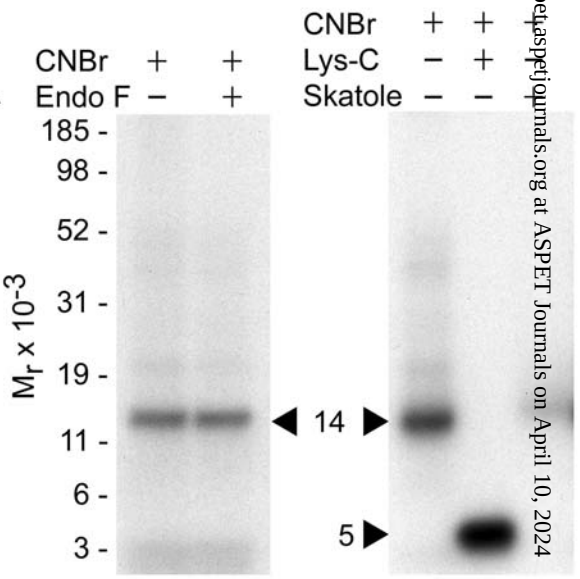

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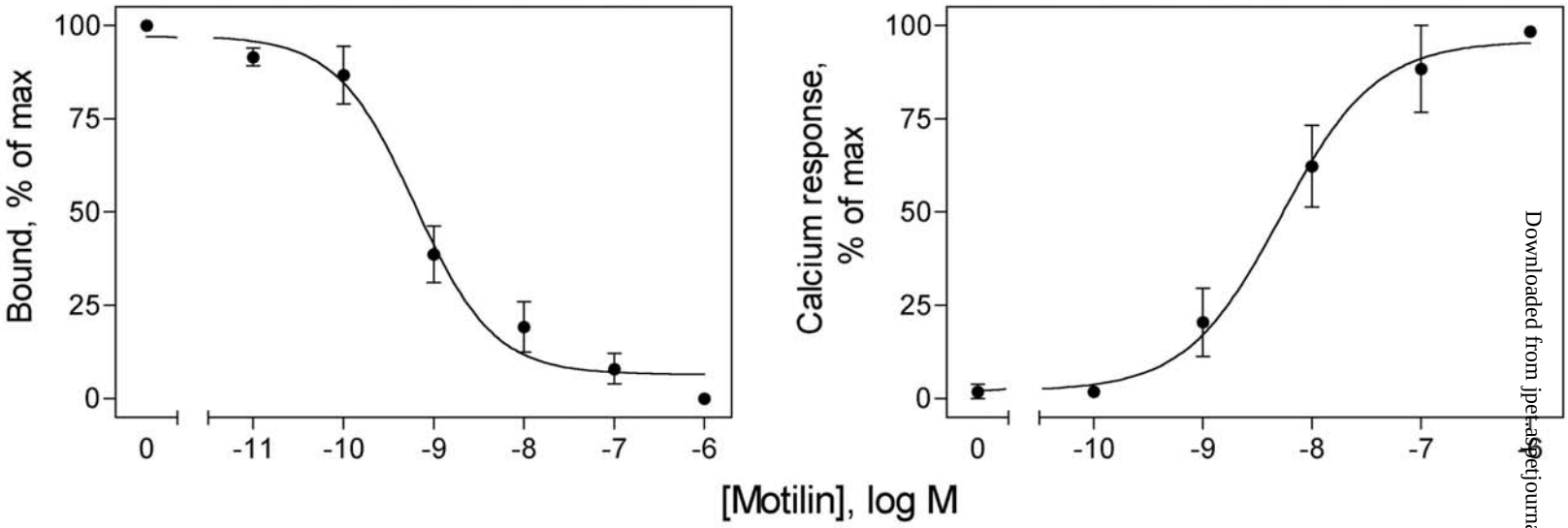
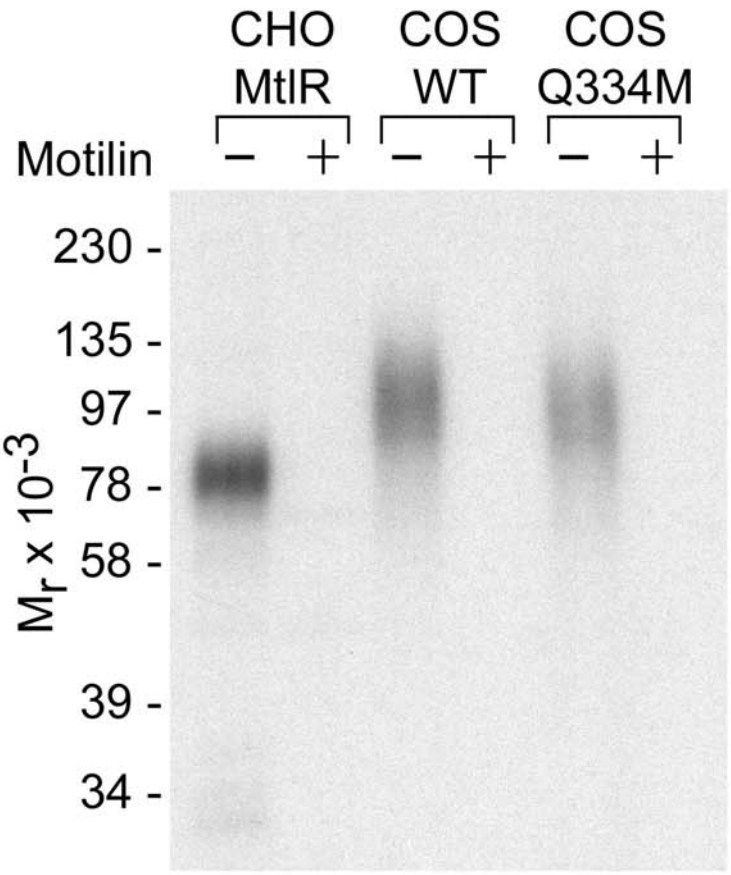
Figure 4





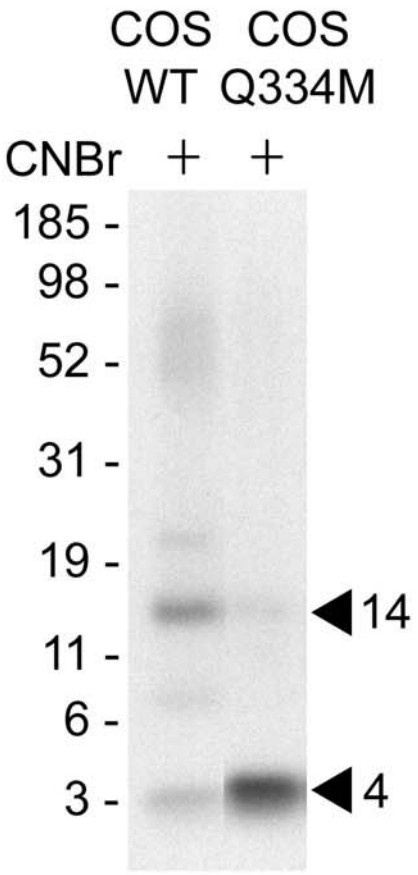


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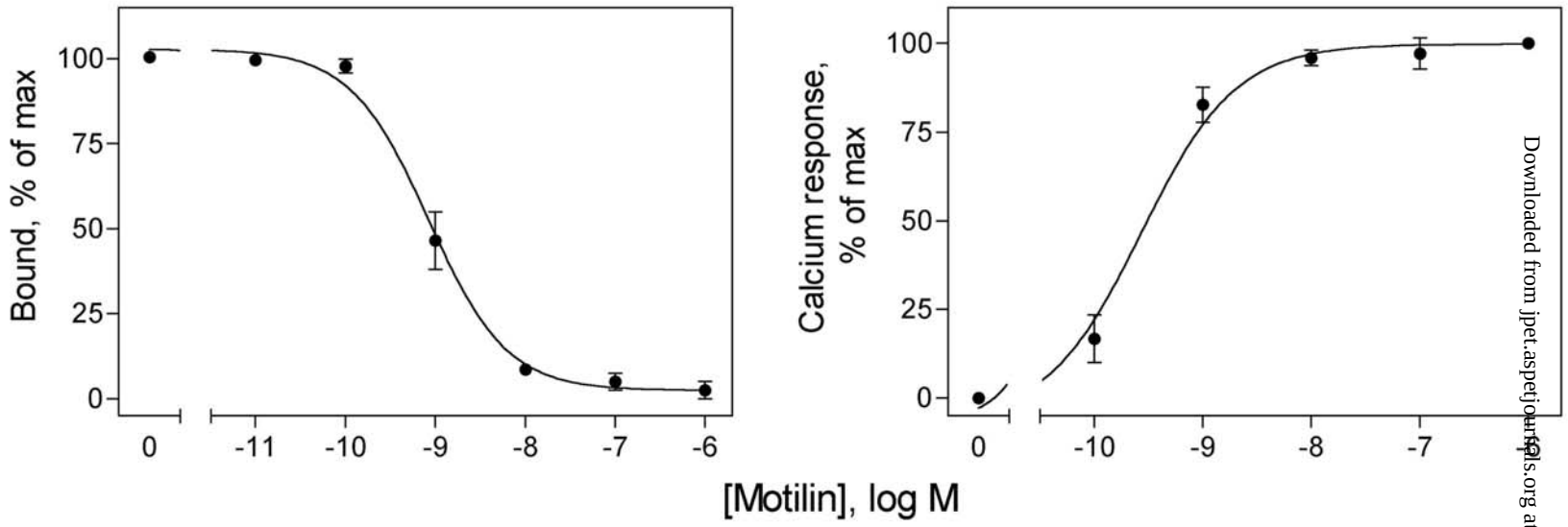
Figure 5



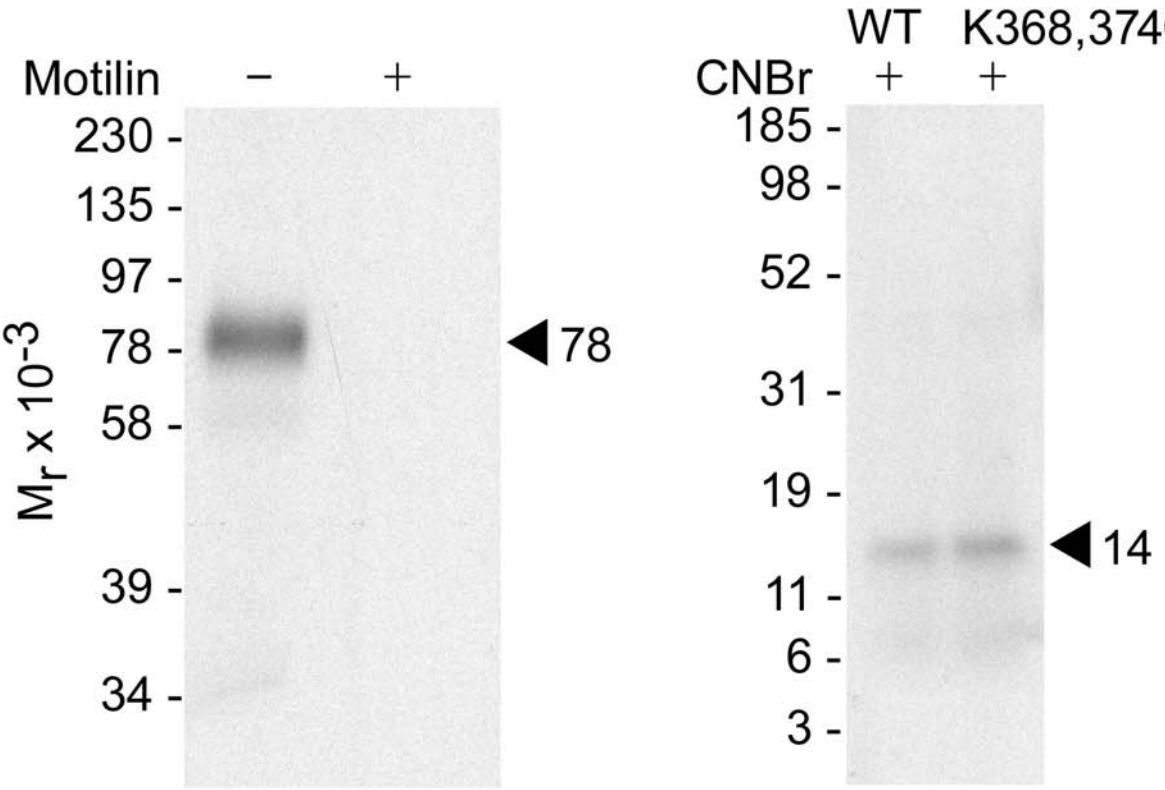


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Figure 6