Published in final edited form as:

Biochemistry. 2016 March 29; 55(12): 1772–1783. doi:10.1021/acs.biochem.5b01195.

Structural insights into the activation of human relaxin family peptide receptor 1 by small molecule agonists

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Abstract

The GPCR relaxin family peptide receptor 1 (RXFP1) mediates the action of relaxin peptide hormone including its tissue remodeling and antifibrotic effects. The peptide hormone has a short half-life in plasma, limiting its therapeutic utility. However, small molecule agonists of human RXFP1 can overcome this limitation and may provide a useful therapeutic approach especially for chronic diseases such as heart failure and fibrosis. The first small molecule agonists of RXFP1 were recently identified from a high throughput screen using a homogenous cell-based cAMP assay. Optimization of the hit compounds resulted in a series of highly potent and RXFP1 selective agonists with low cytotoxicity, and excellent *in vitro* ADME and pharmacokinetic properties. Here we undertook extensive site-directed mutagenesis studies in combination with computational modeling analysis to probe the molecular basis of the small molecule binding to RXFP1. The results showed that the agonists bind to an allosteric site of RXFP1 in a manner that closely interacts with the seven transmembrane domain (TM7) and the third extracellular loop (ECL3). A number of residues were found to play an important role in the agonist binding and receptor activation including a hydrophobic region at TM7 consisting of W664, F668 and L670. The G659/T660 motif within ECL3 is crucial to the observed species selectivity of the agonists for RXFP1. The receptor binding and activation effects by the small molecule agonist ML290 were compared with the cognate ligand relaxin peptide, providing valuable insights on the structural basis and molecular mechanism of activation and selectivity of the agonists for RXFP1.

The relaxin family peptide receptor 1 (RXFP1) is a member of class A relaxin/insulin-like family of GPCR receptors.¹ Relaxin hormone is the endogenous ligand that binds to RXFP1

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The authors declare no competing financial interest.

Supporting Information

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and activates various signaling pathways including cAMP signaling, which then induces a variety of biological responses. Another member of this receptor family is RXFP2, a receptor for insulin-like 3 peptide.^{2, 3} Both RXFP1 and RXFP2 receptors contain a large extracellular domain with a single low-density lipoprotein class A (LDLa) module at the amino terminus and ten extracellular leucine-rich repeat domains (LRRs) followed by seven transmembrane (7TM) helical domains. The LRRs and the first two exoloops of the transmembrane domains participate in relaxin binding, whereas the LDLa module is required for receptor activation.^{4, 5}

The wide range of effects and applications of relaxin underscores the importance of this hormone in human physiology and disease.⁶ Several clinical trials have tested recombinant human relaxin hormone as a treatment for scleroderma, cervical ripening, fibromyalgia, preeclampsia, and acute heart failure.⁷ However, the recombinant hormone has a very short half-life *in vivo*. Small-molecule agonists of RXFPs have attracted great interest in the past years as valuable tools in furthering the understanding of relaxin hormone biology and having the potential for wider therapeutic applications.

High throughput screening of human RXFP1 enabled us to identify the first series of small-molecule agonists of RXFP1 reported to date.⁸ Optimization of the hit compounds resulted in a series of highly potent and RXFP1-selective agonists with low cytotoxicity, and excellent *in vitro* ADME and pharmacokinetic properties.⁹ Our previous data suggested that the small molecule agonists bind to a region within 7TM and do not require the LDLa domain for receptor activation.⁹ In this study, we undertook site-directed mutagenesis studies and computational modeling to further characterize the binding mechanism of these small molecule agonists with RXFP1. Binding models of the agonists were probed using an approach combining homology modeling, docking, molecular dynamics (MD) simulations, and binding free energy calculations. The experimental and modeling data suggest that the small molecule agonists bind to a site of the receptor formed primarily by TM6 and TM7, which is non-competitive with relaxin hormone. Comparisons with RXFP2 and other species of RXFP1 revealed that ECL3 plays a crucial role in the receptor activation and species selectivity. In particular, residues G659/T660 likely act as a lock to prevent the loop from changing conformation during human RXFP1 receptor activation. The proposed binding model provides insights into the structural basis of the mechanism-of-action of the small molecule agonists and may be useful for further lead optimization and drug development.

Methods

Homology modeling

Sequences of human RXFP1 and RXFP2 (denoted as hRXFP1 and hRXFP2) as well as RXFP1 from other species including mouse, macaque, pig, and rat were retrieved from UniProtKB database (access code hRXFP1:Q9HBX9, hRXFP2:Q8WXD0, mouse RXFP1:Q6R6I7, macaque RXFP1:I2CTQ4, pig RXFP1:F1RW91, rat RXFP1:Q6R6I6). The transmembrane domain of the RXFPs was modeled using the structure of human β_2 adrenergic receptor β_2 AR as a template. The active conformation was generated utilizing the active form of β_2 AR as a template (PDB entry 3P0G),¹⁰ while the inactive form was built based on the inactive form of 2RH1.¹¹ Multiple sequence alignment and homology

modeling were performed using the MOE program (CCG Inc., 2014) and Modeler.¹² The modeled structures of RXFP1 and RXFP2 were subjected to energy minimization and MD simulations as described below.

Docking of ML290 to hRXFP1

The binding mode of ML290 with the active RXFP1 was predicted using a step-wised ensemble-docking approach^{13, 14} Ensembles of the active RXFP1 structure were generated from the MD simulations. A total of five cluster representatives were selected from the trajectory clustering analysis and used for the following docking study. The AutoDock 4.2 program¹⁵ was applied for the docking of ML290 to ensembles of the RXFP1 structure. The active site of the protein was defined by a grid of $60 \times 60 \times 60$ points with a grid spacing of $0.5 \text{ \AA}$ to encompass the orthosteric as well as potential allosteric binding site for an unbiased docking search. The Lamarckian Genetic Algorithm (LGA)¹⁶ was applied with 100 runs and the maximum number of energy evaluations was set to 2×10^6 . All poses from each ensemble docking were retained. Binding mode analysis was performed using the AutoDock package. The outputs from each ensemble docking were merged and re-clustered with a cutoff of $2.0 \text{ \AA}$ in positional root-mean-square deviation (RMSD). The major clusters were analyzed to identify the plausible binding modes. Three optimal binding models were selected and subjected to energy minimization and MD simulations as described below.

MD simulations and binding free energy calculations

MD simulations of the RXFP1 structural model and the binding complexes with agonists were performed using the Amber 14 and the ff99SB force field.¹⁷ The Lipid 14 force field¹⁸ was used to model the bilayer and the GAFF force field¹⁹ was used for the small molecules. Four structural models were simulated: the active form of RXFP1, the inactive form of RXFP1, the inactive form of RXFP2, and the binding complex of active RXFP1 with agonist ML290. The protein structure was inserted into a hydrated, equilibrated POPC bilayer using the CHARMM-GUI interface.²⁰ The simulated system was neutralized with counter-ions sodium and chloride. The size of the whole system was approximately $84 \times 84 \times 105 \text{ \AA}$ and a total of 150 ~170 POPC molecules. Simulations were heated from 0K to 100 K in the NVT ensemble and then from 100 K to 303 K in the NPT ensemble with harmonic restraints on the protein atoms. Simulations were equilibrated at 303 K in the NPT ensemble and subsequent production simulations were performed using a Langevin thermostat for temperature control while pressure was controlled using the anisotropic Berendsen barostat. Bond lengths involving hydrogen were constrained with SHAKE and the time step for all MD simulations was set to 2 femtoseconds. A non-bonded cutoff of $10 \text{ \AA}$ was used, and the particle mesh Ewald (PME) method was employed to calculate the long-range electrostatic interactions. A total of 50 ns of production simulations were performed for each simulated system.

The binding free energies were calculated using the MM-PBSA method.²¹ A set of 250 snapshots were extracted from trajectories of simulated systems at 200 picosecond intervals from the 50-ns of each MD simulations. The polar contribution (G_{PB}) was calculated using the Poisson Boltzmann equation. The nonpolar contributions (G_{SA}) were estimated using the MSMS algorithm according to the equation: $G_{SA} = \gamma \times \text{SASA} + b \text{ kcal/mol}$, with γ and b

set to $0.00542 \text{ kcal/mol} \cdot \text{\AA}^{-2}$ and 0.92 kcal/mol ,²² and the probe radius used to calculate the solvent accessible surface area (SASA) was set to $1.4 \text{ \AA}$. The entropy contribution was neglected in the binding free energy calculations. Decomposition of the calculated binding free energies was performed using the MM-PBSA module in the AMBER 14 package.¹⁷

Site-directed mutagenesis

Full-length human *RXFP1* in pcDNA3.1TM/Zeo(+) AmpR mammalian expression vector (Invitrogen, Carlsbad, CA) containing an N-terminal FLAG tag and a bovine prolactin signal sequence was used as a template for site-directed mutagenesis.²³ The FLAG tag does not interfere with receptor activity.²⁴ Mutant DNA was prepared using long-range PCR with *PfuTurbo*® DNA polymerase (Agilent Technologies, Santa Clara, CA, USA) with overlapping primers containing the mutated nucleotide sites. The template was digested with *DpnI* (Promega, Madison, WI) and the DNA was transformed into XL10-Gold ultracompetent cells (Agilent Technologies). The cDNA of mutant clones was fully sequenced to confirm the presence of the correct mutations and the absence of additional substitutions.

Cell transfection and cAMP assays

The HEK293T cells (ATCC, Manassas, VA) were transiently transfected with wild-type and mutant RXFP1 expression constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were tested in the cAMP assay 48 hr after transfection. Cellular cAMP level was measured using HTRF cAMP HiRange kit (CisBio, Bedford, MA, USA) according to manufacturer's protocol. Cells were stimulated with various concentrations of porcine relaxin peptide (RLN) or ML290. Porcine relaxin peptide was a gift from Dr. O. David Sherwood (University of Illinois at Urbana-Champaign).²⁵ The signal was read on a FLUOstar Omega (BMG Labtech, Cary, NC) plate reader. All experiments were repeated at least three times in triplicates. Ligand activity is reported through two measurements: EC₅₀ (concentration necessary to reach 50% of the maximum cAMP signal produced by the molecule) and maximum response (E_{max}, corresponding to the level of cAMP elevation normalized to FSK control). Statistical processing of the data was performed using GraphPad Prism software (San Diego, CA).

RXFP1 cell surface expression assay

Cell surface expression of RXFP1 constructs was determined using flow cytometry as previously described.²⁶ Briefly, 24 hours after transfection with RXFP1 construct or empty vector pCR3.1, the HEK293T cells were harvested in PBS/5 mM EDTA. Cells were fixed in stain buffer (2% BSA/PBS) containing 3.7% formaldehyde, washed, and incubated with $0.5 \mu\text{g}$ anti-FLAG M1 Ab (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 4°C . After washing, the cells were incubated with $1 \mu\text{g}$ Alexa Fluor 488 goat anti-mouse IgG (Life Technologies, Grand Island, NY) for 20 minutes at 4°C . Cells were washed and resuspended in stain buffer for analysis on an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Cells transfected with an empty vector were used as the background cut-off to determine RXFP1 expression. The expression of the mutant receptors was normalized to the expression of wild-type receptor determined in parallel experiment. All experiments were performed in triplicate and analyzed using GraphPad Prism software.

Results and Discussion

Structural model of hRXFP1

The 7TM domain of hRXFP1 shares a sequence identity with other GPCRs of between 12–17%, with 20–28% for the transmembrane helices and 5–11% for the loops. A multiple sequence alignment of hRXFP1 with agonist-bound (active) and antagonist-bound (inactive) structures of GPCRs indicated that the human β_2 adrenergic receptor (β_2 AR) shares the highest sequence identity with hRXFP1 (27% in TM and 10% in loops) (Figure S1). Therefore, we modeled both the active form and inactive form of hRXFP1 using the structures of β_2 AR as a template (PDB 3P0G and 2RH1) (Figure 1). The models were constructed and refined with a step-wise energy minimization and MD simulations. Applying a similar approach in the GPCR Dock 2013 Assessment, this method achieved the best accuracy in the TM bundle structure prediction category.²⁷

Comparison of the hRXFP1 model with β_2 AR and other GPCRs revealed both commonalities observed in other GPCRs as well as several distinct structural features of hRXFP1. First, while the structure of the internal region within the 7TM, the typical orthosteric site for most druggable GPCRs, is similar between β_2 AR and hRXFP1, there are important differences regarding their physical properties. For example, the polar residues S203, S204, S207 within TM5 of β_2 AR, which are involved in ligand binding, are replaced with nonpolar residues (I583, F584, and I587) in hRXFP1. Other ligand-interacting residues in β_2 AR such as D113 (TM3) and W316 (TM7) are also replaced by non-polar residues A492 and P671 in hRXFP1. Therefore, the internal region between the 7TM of hRXFP1 is dominated by hydrophobic interactions. Second, hRXFP1 ECL2 does not possess the two cysteine residues which are typically observed in GPCRs and form a β -hairpin stabilized by a disulfide bond. Consequently, ECL2 is more dynamic in the structural models when compared to other GPCRs. As hRXFP1 possesses a “lid” LRR domain and the interface between the TM domain and LRR domain is presumably the binding site for relaxin hormone, the high flexibility of ECL2 is likely necessary to facilitate relaxin binding and receptor activation. Third, the toggle switch residues W641 and F645 are conserved in hRXFP1 but appear to adopt a different conformation that positions them more deeply in the binding pocket than in other GPCR structures. The “ionic lock” motif (E/DRY) is also generally conserved,²⁸ though the highly conserved residue arginine is replaced with a Lys in hRXFP1 (E509/K510/Y511). Similar to the template structures of β_2 AR, the ionic bonds in both the active and inactive form of hRXFP1 models are broken. Compared to the R135-E247 ionic interaction in the inactive form of rhodopsin,²⁹ the ionic interaction between K510-E623 in hRXFP1 is not favorable.

Finally, comparison of the active and inactive form of hRXFP1 structural models showed that the outward movement of TM6 and TM7 at the cytoplasmic face is similar to the template structures of β_2 AR. However, the inward movement of ECL3 in the active form of hRXFP1 appeared to be more profound, while the conformational change of ECL3 in the active and inactive forms of β_2 AR was limited (Figure 1). Molecular dynamics simulations indicated that the 7TM of both the active and inactive forms of the hRXFP1 structural models remained rather stable during a time course of 50-ns simulations in the bilayer

system, and the intracellular and extracellular loop regions exhibited a more dynamic structure (Figure 2). Interestingly, ECL3 in the active form appeared to be more dynamic than the inactive form, indicating that the receptor likely favors an inactive conformation in the apo state and undergoes conformational changes to the active conformation upon ligand binding. As shown in Figure 2, ECL3 was significantly stabilized by small molecule binding in the active form.

Binding interaction of ML290 with hRXFP1

We modeled the binding interactions of the agonist ML290 with hRXFP1 using an ensemble-based hierarchical docking protocol to incorporate protein flexibility (Figure S2 and S3).¹³ Clustering analysis of docking poses showed that the small molecule binds to the 7TM site of hRXFP1 in a similar manner as other agonists bound to different GPCRs. As shown in Figure 3, the core 2-acetamido-N-phenylbenzamide is accommodated in the typical ligand-binding pocket by forming extensive van der Waals and hydrophobic interactions with residues mainly from TM5 and TM7. In particular, the A ring of ML290 points toward a hydrophobic region with residues I583 and I587 in TM5, I493 and T496 in TM3, and F645 and K648 in TM6. On the other side, the B ring moves upward more close to TM7 and forms a well-defined aromatic stacking interaction network with residues W664, F668, and F459. Obviously, the agonist ML290 binding to hRXFP1 is predominantly driven by hydrophobic interactions. The lack of polar and hydrogen bonding interactions within the binding site is uncommon compared to other GPCR agonist binding sites.³⁰ In addition, ML290 adopts the same intramolecular hydrogen-bonding conformation in the binding pocket as is observed in the crystal structure of the isolated small molecule and by solution NMR.⁹

Remarkably, the trifluoromethylsulfonyl group of ML290 interacts with the two hallmark residues G659/T660 at the C-terminus of ECL3. As reported in our previous studies, the G659/T660 to D659/S660 substitution within ECL3 of hRXFP1 completely abolished the activation by ML290.⁹ The sulfonyl oxygen atom forms a hydrogen bond with the backbone of Thr-660 in the binding model (Figure 3). MD simulations showed that ECL3 in the agonist-bound form was significantly stabilized by ML290 (Figure 2). Since the flexibility of ECL3 is associated with the conformational changes of TM6 and TM7, the close interactions of the small molecule with ECL3 and the G659/T660 motif suggest an important functional role in triggering the RXFP1 activation.

SAR analysis of ML290 derivatives bound in hRXFP1

A number of ML290 derivatives have been evaluated in our previous efforts to optimize the hit compound from a high throughput screen.⁹ Modified compounds with different substituents showed potencies ranging from 2 μ M to 50 nM and varying ability to activate the receptor (Figure 4), providing an excellent testing set for the predicted binding models. As expected, the analogs adopt the same conformation in the binding site of hRXFP1 as observed with ML290 (Figure 5). An intramolecular H-bond was formed within the docked conformation of compound 4, and showed a significant increase of binding free energy in comparison with compound 3. The addition of an alkoxy group in ortho-position apparently stabilized the binding conformation of the small molecule in the pocket, and therefore

significantly enhanced the binding affinity. More promisingly, the calculated binding free energies of these derivative compounds showed a good correlation with the experimental data EC_{50} ($R^2 = 0.89$) (Figure 4), supporting the predicted binding model with hRXFP1.

A major modification of initial hit compound 1 identified in qHTS is the aniline ring at the meta-position. Substituents at this position for compounds 4–6 (SO_2CF_3 , $F=0.73$; SCF_3 , $F=0.35$; and CF_3 , $F=0.38$) provided increasingly potent activity. As shown in the binding models, this functional group is oriented to ECL3 and interacts closely with the G659/T660 motif, thus playing a driving force in triggering the conformational changes for receptor activation. The CF_3 group can effectively interact with ECL3, while the additional H-bond observed with SO_2CF_3 may explain its increased activity. Modifications of compounds 7–11 on the A ring introduce longer alkoxy aliphatic chains in *ortho*-position. The optimized compound 11 exhibits a remarkable increase in activity from micromolar EC_{50} s of the initial hits to a potency of 47 nM. An examination of the binding model shows that this aliphatic chain is inserted into a hydrophobic pocket region formed mainly by residues I493, Y579, and I583 between TM5 and TM6, which likely contributes to increased binding affinity (Figure 5).

Site-directed mutagenesis

Based on the binding model analysis, we conducted extensive site-directed mutagenesis studies to probe the mechanism of ML290 binding with hRXFP1. A total of 18 residues were selected for mutation analysis based on a binding free energy decomposition analysis (Figure 6). Both the mutants and the wild-type (WT) receptor were tested for RLN and ML290 activation using the cAMP assay in a concentration-response manner, thus providing a direct comparison of the mutational effects associated with the small molecule agonist and RLN (Table 1, Figure 7A). The surface expression of these mutants was also measured to compare WT and mutant receptors expression on the cell membrane (Figure 7B). Expression was highly variable, possibly due to differences in transfection efficiency. However, mutant receptors with >40% expression had E_{max} similar to wild-type, which suggests mutant receptors with at least 40% wild type expression induced a full response.

Five residues (T496, A492, I493, I583, and K510) in TM3 and TM5 were examined. T496 is particularly interesting as it is located at the bottom of the binding pocket and formed hydrophobic interactions with the phenyl ring of ML290. It is possible that the side chain of T496 forms a hydrogen bond interaction with the amide group of ML290, though it may not contribute significantly to binding energy. Three mutants of the residue (T496A/T496E/T496F) were tested to probe its binding interaction. The T496A mutation did not affect RLN binding and activation of RXFP1, but had a 10-fold increased potency for ML290. Similarly, the T496F mutation did not affect RLN activity but caused an 8-fold increase in potency for the small molecule agonist. In contrast, the T496E mutant was significantly less active with both RLN and ML290 (Table 1). These results suggest that residue T496 plays an important role in the agonist binding through hydrophobic binding interactions, since increasing the hydrophobicity of this residue increased small molecule binding affinity.

Another mutant A492S, which is near to T496, did not show significant effects on the agonist and RLN activation. However, the mutations of I493N and I583E significantly

decreased the EC₅₀ and efficacies for both RLN and ML290. In the case of I493N, poor cell surface expression contributes to the observed changes in activity. Both residues, along with I587, V542, and Y579 in TM4 and TM5, form a hydrophobic pocket that possibly accommodates the alkoxy aliphatic group observed in the SAR study (Figure 4). Changes of hydrophobic residues to polar and charged residues apparently impair both ligand binding and receptor activation.

K510 is a putative ionic lock residue at the intracellular end of TM3. In other GPCRs this is a highly conserved arginine residue. The K510A mutation did not show a significant impact on activation by both RLN or ML290. As observed in the modeled structure, the ionic lock interactions in hRXFP1 appeared to be unfavorable in the MD simulations, suggesting that it may not play an important role in receptor activation for hRXFP1 in particular.

Four TM6 residues (W641, I644, F645, and K648) were evaluated. Mutation of W641A and F645A that act as a toggle switch residues for receptor activation in other GPCRs did not show significant effects on either RLN or ML290. Mutant F645E impaired RLN and ML290 activation but this could be due to the poor cell surface expression of the mutant. Interestingly, another mutant I644A in the proximity of W641 showed a 3-fold decreased potency for ML290. The mutation K648E significantly affected RLN activation, but had only a slight impact on activation by ML290. K648 is located on top of the binding pocket. Residues at this position such as asparagine in β 2AR or histidine in D3 typically participate in ligand binding. K648 in hRXFP1 model pointed upward to the interface of the TM and LRR domain with high flexibility. It is likely that it contributes as a gating residue at the ligand binding interface to facilitate RLN binding or the LRR domain closure for receptor activation.

TM7 is extensively involved in binding interactions with ML290 in the binding model. We tested four residues within this domain: W664, I667, F668 and L670. Except for L670, the other three residues are predicted to participate in agonist binding by forming extensive hydrophobic and aromatic stacking interactions (Figure 3). L670 is positioned deeply in the binding pocket, adjacent to the toggle switch region. Mutations of W664A, F668A and L670A dramatically impaired agonist binding and activation; however, I667E had poor surface expression, confounding further interpretation. Moreover, all the mutations affected RLN activation significantly, suggesting that these residues also play an important role in relaxin peptide binding and receptor activation.

Three residues within ECL2 (F564A, H567A, E569A) were mutated to test whether they also participate in agonist binding. While H567A and E569A did not show significant effects on either ML290 or RLN, F564A dramatically decreased their activities. The same result was reported recently by Diepenhorst *et al.*³¹ However, surface expression of the mutant was low in our study (Figure 7B). It is unclear that whether the inactivity resulted from a binding effect or low receptor expression. F564 forms a stacking interaction with the phenyl ring of ML290 in the predicted binding model, therefore, it is possible that mutation of phenylalanine to alanine decreased ML290 binding affinity and its agonist activity.

Finally, we examined the most interesting motif G659/T660 within the ECL3. As expected, mutant G659D completely abolished the activity of ML290 but had no impact on RLN activation. On the other hand, T660A did not show a significant effect on either ML290 or RLN stimulation (Table 1). These results are consistent with the predicted binding model that ML290 forms H-bonding with the backbone of T660, therefore, mutation of T660A had no effect on the binding activity.

Structural basis of selectivity to hRXFP1

Experimental studies previously demonstrated that ML290 is more than 100-fold selective toward hRXFP1 over hRXFP2.⁹ The two RXFP members possess 80% sequence similarity within the 7TM domain. To investigate the structural basis of selectivity, we generated a structural model of hRXFP2, and docked ML290 to the binding site using the same ensemble-based docking approach. As shown in Figure 8A, the small molecule adopted a binding mode at the allosteric site of hRXFP2 in the same manner as observed with hRXFP1. Key interacting residues that are involved in hRXFP1 binding interactions with the agonist, such as T506, F574, W674, I677, F678, are conserved in hRXFP2. However, there was a striking difference in the G659/T660 motif within ECL3 of hRXFP1. In hRXFP2 this motif is replaced with D669/T670. As shown above, a single hRXFP1 mutation G669D can completely abolish activation by ML290, indicating that this residue plays a pivotal role in the agonist binding and receptor activation. Residue D669 points toward the agonist binding site in the binding complex of hRXFP2 with ML290. MD simulations showed that it forms a potential H-bond with Y577 in ECL2. Consequently, ECL3 of hRXFP2 was more stable than that of hRXFP1. It is possible that D669 introduces a steric hindrance to ML290 binding and prevents the small molecule from making close interactions with ECL3 for receptor activation. In fact, we tested one of the ML290 derivatives with RXFP2/INSL3 in an antagonistic mode and the compound was an antagonist of hRXFP2 (unpublished data).

In addition to hRXFP2 selectivity, the small molecule agonist also demonstrates species selectivity. The mouse and rat RXFP1 are not activated by ML290, but monkey and pig RXFP1 are fully activated.³² Sequence alignment shows that monkey and pig RXFP1 have the same e GT motif within ECL3 as hRXFP1, whereas mouse and rat RXFP1 contain residues DS/T at that position. Again, this finding reiterates the importance of the GT motif in receptor activation. We previously tested a number of chimeric mouse/human RXFP1 constructs and found that the substitution of mouse ECL3 sequence with the human ECL3 resulted in full activation of the mouse receptor.⁹ Further structural analysis of the mouse RXFP1/ML290 binding model indicated that, while the interacting residues in the binding pocket are conserved between mouse and human RXFP1, residue D659 in an inactive form of mouse RXFP1 is capable of forming an intramolecular H-bond with residue Thr-662 in TM7 (Figure 8B). Therefore, another possibility is that D659 may play a role in ligand activation by locking the receptor in an inactive state.

Proposed mechanism-of-action of RXFP1

A two-site binding model of RLN binding to RXFP1 was proposed by Hals *et al* and asserts that the relaxin peptide binds to both the high-affinity LRRs within receptor ectodomain and the lower affinity 7TM binding site, resulting in the conformational changes and closure of

the ectodomain during activation.^{4, 6} According to the ligand binding and activation model, the interface between the LRR domain and the TM domain serves as the orthosteric binding site. The cognate ligand relaxin first interacts with the LRR domain, and then with the extracellular loops of the TM domain, mainly with ECL2.³¹ Similar to other GPCRs, this causes the conformational changes of TM6 and TM7 followed by receptor activation.

As previously reported, ML290 is non-competitive with the relaxin ligand for receptor binding.⁹ Here, through molecule modeling and mutagenesis studies we demonstrated that the agonist binds to an allosteric site in the binding pocket of 7TM domain, mainly interacting with the TM7 and ECL3. While the binding of ML290 is predominantly driven by hydrophobic interactions at the allosteric binding site of hRXFP1, specific interactions with ECL3 and a patch of residues in TM7 effectively trigger the conformational changes necessary for receptor activation (Figure S4). The residues W664/I667/F668 at the extracellular end of TM7 also affect RLN binding and activation significantly, suggesting that this region is involved in the binding interaction with RLN. It is worth mentioning that the mutants of the toggle switch residue W641 and the putative ionic lock residue K510 do not alter ligand binding and receptor activation for either the agonist or RLN. Given that RXFP1 has a LRR domain that is essential for receptor activation, it is possible that the toggle switch of RXFP1 is less important as receptor activation is triggered by more profound domain movements within the receptor.

One of the striking findings revealed by our mutagenesis data is that residues G659/T660 within ECL3 plays a pivotal role in receptor activation and selectivity by the small molecule agonist (Figure 9). Modeling and molecule dynamic simulations showed that the loop ECL3 is rather flexible and undergoes conformational changes in the inactive form, whereas the characterized GT motif is capable of forming potential H-bond interactions with the agonist to stabilize the active form. Unlike other GPCRs where the long ECL2 is more involved in agonist binding,³⁰ the interactions of the ECL3 in hRXFP1 with the small molecule agonist play a crucial role for receptor activation. As the ECL2 of hRXFP1 more likely participates in domain interactions with the LRR, the ELC3 at the domain interface might function as a “flexible activation loop” to facilitate relaxin and agonist binding.

Conclusion

In this study we have examined the binding interaction and activation mechanism of the small molecule agonist ML290 to its RXFP1 receptor through extensive site-directed mutagenesis in combination with structural modeling studies. We have shown that the small molecule agonists bind to an allosteric site of RXFP1 in a manner that closely interacts with the helix 7 of 7TM domain and the extracellular loop ECL3. In particular, we have demonstrated that residue motif G659/T660 within ECL3 is crucial in explaining the observed species selectivity of the compounds for RXFP1 over other related receptors. These results reveal valuable insights into the structural basis of receptor activation and selectivity by the small molecule agonists, and provide a useful guide for further structure-based drug design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Source Statement. This research has been funded in part by the Florida Department of Health, James and Esther King Biomedical Research Program grant 3KFO1, and National Cancer Institute grant 1U01CA177711 (A.I.A). B.A.H. was supported by NIH/NIGMS T34GM083688 fellowship.

The authors thank Dr. O. David Sherwood, University of Illinois, for providing porcine relaxin.

ABBREVIATIONS

ADME	absorption, distribution, metabolism, and excretion
β₂AR	β ₂ adrenergic receptor
cAMP	cyclic adenosine monophosphate
ECL1-3	extracellular loops 1-3
LDLa	low-density lipoprotein class A module
LRRs	leucine-rich repeat domains
NMR	nuclear magnetic resonance
RXFP1	relaxin family peptide receptor 1
TM1-7	transmembrane domains 1-7

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Figure 1.

(A) Structural models of human RXFP1(hRXFP1). The active conformation is shown in green and the inactive conformation is shown in blue. The TM6 and ECL3 of the active and inactive form of hRXFP1 are shown in red and dark yellow. Key residues in the binding pocket of the 7TM domain are shown in a separate panel. (B) Sequence alignment of the TM6/TM7 and ECL3 of hRXFP1 with other four GPCRs: human β 2 adrenergic receptor (hB2AR), turkey β 1 adrenergic receptor (tB1R), human A2A adenosine receptor (hA2AR), bovine rhodopsin receptor (bRhoR).

Figure 2. MD simulations of the hRXFP structural models. (A) RMSDs of the backbone atoms of hRXFP1 in the apo and ligand-bound forms during the 50-ns simulations. (B) RMSDs of the backbone atoms of the extracellular loop ECL3 with respect to their starting structure during the 50-ns simulations.

Figure 3.

Binding interaction of ML290 with hRXFP1. The agonist ML290 is shown in stick (carbon atoms in cyan, oxygen and nitrogen atoms are shown in red and blue). Key residues within hRXFP1 involved in binding interaction are shown in green/yellow/magenta (carbon atoms). The two phenyl rings of ML290 are labeled as A and B on the right panel. The β_2 AR agonist BI-167107 (PDB code 3P0G) is superimposed in the binding pocket of hRXFP1 and shown in yellow on the left panel.

Figure 4.

(A) Derivatives of the hRXFP1 agonist ML290 and predicted binding energies from docking study. (B) Plot of correlation between experimental EC₅₀'s and predicted binding energy ($R^2 = 0.89$).

Figure 5. Predicted binding models of derivatives of ML290 agonist within hRXFP1. Key residues of hRXFP1 are shown in stick and the two regions are circled in red and blue. The hydrophobic pocket in the binding region A is depicted in a surface representation on the left panel.

Figure 6.
(A) 2D diagram of binding interaction of ML290 within hRXFP1. (B) Binding free energy decomposition of key residues of hRXFP1 involved in binding interaction with ML290.

A

B

Figure 7. Site-directed mutagenesis results. (A) The maximum response of hRXFP1 mutants stimulated by relaxin (RLN) and ML290. The activity of cAMP elevation was normalized to FSK control. (B) Surface expression of hRXFP1 mutants in transfected HEK293T cells. Data was normalized to wild-type (WT) expression. (C) mutant G659D dose-response activity by relaxin and ML290 in comparison with the wild-type hRXFP1. (D) mutant T660A dose-response activated by relaxin and ML290 in comparison with the wild-type hRXFP1.

Figure 8.

(A) Binding interaction of ML290 within hRXFP2. ML290 is shown in stick. The extracellular loop ECL3 is shown in magenta color. Key residues are shown in green stick. (B) Binding interaction of ML290 within mouse RXFP1 (mRXFP1). An intramolecular hydrogen bond is formed between residue D659 and T662, which may play a role in ligand activation by locking the receptor in an inactive state.

Figure 9.
Proposed Binding Model & Activation of Mechanism of hRXFP1 by agonist ML290.

Summary of relaxin (RLN) and ML290 on mutant receptors. Activities are reported through two measurements: EC₅₀ (concentration necessary to reach 50% of the maximum cAMP signal produced by the molecule and maximum response (E_{max}, corresponding to the level of cAMP elevation normalized to FSK control). The data of mutants (means ± SD) were measured from 3 replicates and the control wild-type were tested with all mutants.

Table 1

Mutation	Position	Relaxin (RLN)			ML290		
		EC ₅₀ (nM)	E _{max}		EC ₅₀ (μM)	E _{max}	
Wild-type	/	0.075 ± 0.016	90.06 ± 2.71		0.648 ± 0.178	76.73 ± 4.32	
A492S	TM3	0.118 ± 0.010	85.68 ± 5.08		0.850 ± 0.401	80.00 ± 5.87	
I493N	TM3	4.561 ± 0.459	42.74 ± 16.40		3.529 ± 0.474	36.62 ± 19.12	
T496A	TM3	0.048 ± 0.009	89.65 ± 1.32		0.056 ± 0.043	87.26 ± 2.82	
T496F	TM3	0.097 ± 0.012	75.77 ± 2.12		0.069 ± 0.011	79.33 ± 1.74	
T496E	TM3	2.436 ± 2.331	53.03 ± 21.23		2.609 ± 1.739	21.26 ± 13.45	
K510A	TM5	0.241 ± 0.213	82.38 ± 4.87		1.281 ± 0.208	57.35 ± 3.85	
I583E	TM5	2.524 ± 1.328	58.49 ± 6.98		4.169 ± 3.560	30.56 ± 6.85	
W641A	TM6	0.076 ± 0.024	82.65 ± 4.90		0.107 ± 0.062	85.89 ± 6.92	
I644A	TM6	12.80 ± 2.814	51.29 ± 22.06		1.739 ± 0.750	44.15 ± 10.51	
F645A	TM6	0.099 ± 0.055	88.23 ± 0.63		0.169 ± 0.018	85.30 ± 1.05	
F645E	TM6	Inactive	25.35 ± 26.12		Inactive	15.80 ± 11.98	
K648E	TM6	Inactive	17.42 ± 2.71		1.241 ± 0.299	49.83 ± 3.22	
W664A	TM7	Inactive	3.15 ± 15.25		Inactive	7.71 ± 16.21	
I667E	TM7	Inactive	13.97 ± 5.38		1.824 ± 1.421	33.41 ± 5.64	
F668A	TM7	Inactive	33.90 ± 28.76		Inactive	17.29 ± 15.51	
L670A	TM7	0.417 ± 0.187	86.89 ± 5.11		1.626 ± 0.813	59.96 ± 7.21	
F564A	ECL2	Inactive	4.47 ± 2.89		Inactive	15.28 ± 16.86	
H567A	ECL2	0.776 ± 0.463	76.41 ± 17.86		0.871 ± 0.106	64.73 ± 16.71	
E569A	ECL2	0.155 ± 0.099	76.39 ± 23.70		0.805 ± 0.377	60.82 ± 24.33	
G659D	ECL3	0.072 ± 0.013	86.83 ± 8.96		Inactive	17.49 ± 12.73	
T660A	ECL3	0.166 ± 0.106	87.36 ± 5.79		0.607 ± 0.086	78.94 ± 5.11	