Structural basis for R-spondin recognition by LGR4/5/6 receptors

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Xenopus Wnt signaling was established by the identification of RSPOs and Yoon 2012). The functional link between RSPOs and Lee 2011; Cruciat and Niehrs 2012; de Lau et al. 2012; Jin and Yoon 2012). They have pleiotropic functions in development and stem cell growth by strongly enhancing Wnt pathway activation, Recently, leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4), LGR5, and LGR6 have been identified as receptors for RSPOs. Here we report the complex structure of the LGR4 extracellular domain (ECD) with the RSPO1 N-terminal fragment (RSPO1-2F) containing two adjacent furin-like cysteine-rich domains (FU-CRDs). The LGR4-ECD adopts the anticipated TLR horseshoe structure and uses its concave surface close to the N termini to bind RSPO1-2F. Both the FU-CRD1 and FU-CRD2 domains of RSPO1 contribute to LGR4 interaction, and binding and cellular assays identified critical RSPO1 residues for its biological activities. Our results define the molecular mechanism by which the LGR4/5/6 receptors recognize RSPOs and also provide structural insights into the signaling difference between the LGR4/5/6 receptors and other members in the LGR family.

Supplemental material is available for this article.

Received April 7, 2013; revised version accepted May 21, 2013.

The R-spondin (RSPO) family of secreted proteins [RSPO1–RSPO4] has pleiotropic functions in development and stem cell growth by strongly enhancing Wnt pathway activation. Recently, leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4), LGR5, and LGR6 have been identified as receptors for RSPOs. Here we report the complex structure of the LGR4 extracellular domain (ECD) with the RSPO1 N-terminal fragment (RSPO1-2F) containing two adjacent furin-like cysteine-rich domains (FU-CRDs). The LGR4-ECD adopts the anticipated TLR horseshoe structure and uses its concave surface close to the N termini to bind RSPO1-2F. Both the FU-CRD1 and FU-CRD2 domains of RSPO1 contribute to LGR4 interaction, and binding and cellular assays identified critical RSPO1 residues for its biological activities. Our results define the molecular mechanism by which the LGR4/5/6 receptors recognize RSPOs and also provide structural insights into the signaling difference between the LGR4/5/6 receptors and other members in the LGR family.

Results and Discussion

Overall structure of the complex

Sequence annotation of full-length human RSPO1 in the UnitProtKB database (entry code Q2MKA7) shows that the FU-CRD1 and FU-CRD2 domains consist of residues Ala34–Asp85 and Met91–Ala135, respectively. Therefore, we expressed and purified human a RSPO1 fragment [Ala34–Ala135] in baculovirus-infected insect cells and named it RSPO-2F. It bound to the human LGR4-ECD.

Keywords: Wnt signal; complex; ligand/receptor interaction

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Article published online ahead of print. Article and publication date are online at http://www.genesdev.org/cgi/doi/10.1101/gad.219360.113.

[1]Received April 7, 2013; revised version accepted May 21, 2013.

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for receptor binding. Consistently, most of the missense importance in fixing the conformation of this region served in all human RSPOs (Fig. 2B), indicating their 2C). The cysteines in the FU-CRDs are strictly con-
truction in protein expression construct design (Ala34–
F4, F5, and F6 in FU-CRD2) (Fig. 2B). The F6 finger connecting loops fixed by disulfide bonds (Fig. 2A). These fingers can be grouped into two types based on different disulfide bond links. In one type (C2), adopted by F1 and F4, two disulfide bonds link four cysteines in the pattern of Cys1–Cys3 and Cys2–Cys4 (Fig. 2A,B). In the other type (C1), adopted by F2, F3, and F5, a single disulfide bond contributes to the conformation constraint by linking the N and C termini of the finger (Fig. 2A,B). The RSPO1-2F model and sequence alignment show that sequence annotation of FU-CRDs in the UniprotKB database is not accurate. The FU-CRD1 and FU-CRD2 domains should consist of residues Ala34–Ile95 and Lys96–Ser143 (Fig. 2B), respectively. They adopt the same C2–C1–C1 architecture (F1, F2, and F3 in FU-CRD1, and Cys43 disulfide bonds, are conserved in LGR5 and LGR6 (Fig. 2B, Supplemental Fig. 3). The LRRCT of the LGR4-ECD is composed of two ordered structural motifs (Phe456–Cys476 and Ile520–Gly527), and the disordered Asp477–Ile519 region between them is not built into the final model due to weak electron densities (Fig. 3C). Major secondary structure elements in the first motif (Phe456–Cys476) include a β strand parallel to LRR1 and a subsequent short helix [Fig. 3C]. Primary sequence analysis shows that it can be regarded as a noncanonical CF3 (C-terminal cysteine-containing flanking domain) motif of LRRs [Kajava 1998]. The previously reported canonical CF3 motif containing three cysteines is GPCR-
specific [Supplemental Fig. 4A; Kajava 1998]. However, the amino acid distance between the third Cys476 and the front two consecutive Cys470 and Cys471 in LGR4/5/6 is shorter than in the canonical CF3 motif [Supplemental Fig. 4A]. The second motif (Ile520–Gly527) is at the C-terminal end of the LGR4-ECD and also contains a β strand parallel to the one in the first motif [Fig. 3C]. These two parallel β strands in the LRRCT form an extension of β sheet 2 of the LRR region [Fig. 3C]. There are five conserved cysteines the LRRCT of LGR4/5/6 [Supplemental Fig. 4A]. The last Cys532 was truncated in the expressed and purified LGR4-ECD, the resting four cysteines form two disulfide bonds (Cys470–Cys522 and Cys471–Cys476), and the first one couples two structural elements together [Fig. 3C].

RSPO1–LGR4 interaction

The LGR4-ECD uses the concave surface of the first β sheet to interact with both FU-CRD1 and FU-CRD2 of RSPO1-2F [Fig. 1]. The interacting residues of the LGR4-ECD are from the LRR3–9 modules, while interacting
residues of RSPO1-2F are from fingers F3, F4, and F5 (Supplemental Table 2). The binding interface can be divided into two subinterfaces based on the involvement of either FU-CRD1 or FU-CRD2 in the binding and their difference in chemical nature. Subinterface I, involving FU-CRD1, primarily consists of salt bridge interactions (Fig. 4A). Acidic Asp85 from the FU-CRD1 F3 finger has salt bridge interactions with basic Arg135 from the LRR4 module of the LGR4-ECD, and basic Arg87 from the same finger has salt bridge interactions with three aspartic acid residues of the LGR4-ECD (Asp137 from LRR4, and Asp161 and Asp162 from LRR5) (Fig. 4A). Subinterface II, involving FU-CRD2, is much more hydrophobic than subinterface I in chemical nature. Phe106 and Phe110 from the F4 finger of FU-CRD2 form a hydrophobic cluster with His157 and Trp159 from LRR5, Ala181 from LRR6, and Val204 and Val205 from LRR7 of the LGR4-ECD (Fig. 4B). His108, Asn109, Lys122, and Arg124 of RSPO1-2F and Glu252, Thr229, Asn226, and Lys251 of the LGR4-ECD are involved in the hydrophilic interactions surrounding the hydrophobic cluster at subinterface II (Fig. 4B).

RSPO1 critical residues for receptor binding and biological activities

Previous results have shown that both FU-CRD1 and FU-CRD2 are necessary for the biological functions of RSPOs [Kazanskaya et al. 2004]. We further explored the roles of specific RSPO1-2F-interacting residues in the abilities of LGR4 binding and Wnt3a-induced STF enhancement. In subinterface I, both Asp85 and Arg87 of RSPO1-2F have salt bridge interactions with the LGR4-ECD (Fig. 4A). Mutation of Arg87 (R87A) decreased the binding affinity of RSPO1-2F with the LGR4-ECD by ~24-fold, from 56.5 nM to 1.32 μM [Fig. 4C, Supplemental Fig. 1A]. The RSPO1-2F R87A mutant also lost nearly all of the ability to increase Wnt3a-induced STF activation (Fig. 4D). On the other hand, mutation of Asp85 (D85A) only brought minor changes in both binding affinity and Wnt3a-induced STF activation [Fig. 4C,D, Supplemental Fig. 1A]. The more critical role of Arg87 compared with Asp85 in receptor binding and biological activities is also supported by sequence alignment showing that Asp85 is replaced with alanine in RSPO2–RSPO4, whereas Arg87 is strictly conserved in all RSPOs [Fig. 2B]. Hydrophobic interactions play more critical roles than hydrophilic interactions at subinterface II. The binding of the RSPO1-2F Phe106Ala mutant (F106A) with the LGR4-ECD was too low to be measured in a surface plasmon resonance (SPR) assay [Fig. 4C, Supplemental Fig. 1A], and this mutant lost all of the ability to increase Wnt3a-induced STF activation (Fig. 4D). The Phe106Ala mutant (F106A) also bound the LGR4-ECD with low affinity (1.31 μM) and did not increase Wnt3a-induced STF activation (Fig. 4C,D, Supplemental Fig. 1A), His108, Asn109, and Arg124, involved in the hydrophilic interactions, are not critical because their mutants exhibited activities very similar to the wild type in both assays [Fig. 4C,D, Supplemental Fig. 1A]. The only exception is Lys122, whose mutation decreased the abilities to bind the LGR4-ECD (0.563 μM) and enhance Wnt3a-induced STF activation (Fig. 4C,D, Supplemental Fig. 1A). These results together elucidate the specific contributions of FU-CRD1 (salt bridge interactions) and FU-CRD2 (hydrophobic interactions) in LGR4/5/6...
receptor binding and the critical roles of conserved Arg87, Phe106, and Phe110 for biological activities of RSPOs (Fig. 2B).

Structural insights into signaling differences between LGR4/5/6 and other members of the LGR family

The closely related LGR4/5/6 receptors constitute a distinct group [B] in the LGR family, and two other groups [A and C] in the same family contain receptors follicle-stimulating hormone receptor (FSHR; LGR1), LHR (LGR2), and TSHR (LGR3) for glycoprotein hormones and LGR7 and LGR8 receptors for relaxin family of ligands, respectively (Hsu et al. 2000; Kong et al. 2010). The ligand recognition and signaling of FSHR, a representative member in group A of the LGR family, have been extensively studied (Fan and Hendrickson 2005; Ulloa-Aguirre et al. 2007), and the complex structure of the FSHR-ECD with its ligand, FSH, has also been determined (Fan and Hendrickson 2005; Jiang et al. 2012). The LGR4-ECD has an architecture similar to the FSHR-ECD, including similar LRRNT, multiple LRR modules (17 in LGR4 and nine in FSHR), and LRRCT. The most significant structure similarity between them exists in the LRRCT region. The LRRCT of the LGR4-ECD consists of two structural motifs (Fig. 3C), and the first one contains a noncanonical CF3 motif (Supplemental Fig. 4A). Similarly, one canonical CF3 motif and one rhodopsin-like extracellular loop in front of the first transmembrane helix have also been observed in the LRRCT [also called a hinge region or signal-specific domain] of FSHR, and their structural arrangement is similar to the two structural elements in the LRRCT of the LGR4-ECD [Supplemental Fig. 4A,B; Jiang et al. 2012]. A difference between them resides in the disulfide bond pattern. In the LRRCT of FSHR, there are six cysteines that form three disulfide bonds to couple the two structural motifs [Supplemental Fig. 4A,B], whereas there is only one disulfide bond coupling the structural motifs in the LRRCT of LGR4 (Fig. 3C; Supplemental Fig. 4A).

The LGR4-ECD and FSHR-ECD are structurally similar, and both use the concave surface of continuous LRR modules (LRR3–9 in LGR4 and LRR1–8 in FSHR) to bind their respective ligands. However, the signal transduction pathways after ligand recognition and receptor activation are different between them. Current experimental evidence indicates that the RSPO signaling mediated by the LGR4/5/6 receptors are not through canonical GPCR signaling pathways (Carmon et al. 2011; de Lau et al. 2011; Ruffner et al. 2012); i.e., as cAMP alteration, Ca$^{2+}$ mobilization, or β-arrestin translocation. This is in contrast to FSHR (LGR1), LHR (LGR2), and TSHR (LGR3), which signal through canonical GPCR signaling pathways upon ligand binding (Birchmeier 2011). Structural and functional studies indicate that full ligand recognition and signaling of FSHR requires a second step interaction of its LRRCT with FSH (Costagliola et al. 2002; Jiang et al. 2012). The initial binding with the LRR region of FSHR reshapes the conformation of FSH to form a pocket. FSHR then inserts its sulfotyrosines from the LRRCT into the FSH nascent pockets, eventually leading to receptor activation and subsequent canonical GPCR signaling pathways (Jiang et al. 2012). The C-terminal part of the LGR4-ECD, including β sheet 2 and the LRRCT, does not interact with RSPO-2F in the complex...
structure [Fig. 1]. Previous functional studies have also shown that deleting the LRRCT region of LGR4 had little effect on RSPO1 activity [Ruffner et al. 2012], and antibodies targeting the C-terminal LRR9-11 modules and LRRCT of LGR5 did not block RSPO1 activity [de Lau et al. 2011]. We also know that the TSR domain and the following positively charged C-terminal tails of RSPOs are dispensable for Wnt/β-catenin signaling enhancement [Kazanskaia et al. 2004; Nam et al. 2006; Kim et al. 2008]. These structural and functional results together suggest that the binding site observed in the complex structure of RSPO1-2F with the LGR4-ECD is the sole one for RSPO recognition by the LGR4/5/6 receptors. The high-affinity one-site binding of RSPOs with the LGR4/5/6 receptors is expected to form a composite platform for interacting with LRPs/6 to direct potential Wnt/β-catenin signaling or with ZNRF3 to inhibit degradation of the Frizzled and LRP5/6 receptors [Supplemental Fig. 5]. The second ligand-binding site involving the LRRCT close to the seven-transmembrane domain of FSHR is necessary for its activation and subsequent signaling through coupled heterotrimERIC G proteins. The absence of this site in RSPO recognition by the LGR4/5/6 receptors is expected to form a composite platform for interacting with LRPs/6 to direct potential Wnt/β-catenin signaling or with ZNRF3 to inhibit degradation of the Frizzled and LRP5/6 receptors [Supplemental Fig. 5]. The second ligand-binding site involving the LRRCT close to the seven-transmembrane domain of FSHR is necessary for its activation and subsequent signaling through coupled heterotrimERIC G proteins. The absence of this site in RSPO recognition by the LGR4/5/6 receptors is expected to form a composite platform for interacting with LRPs/6 to direct potential Wnt/β-catenin signaling or with ZNRF3 to inhibit degradation of the Frizzled and LRP5/6 receptors [Supplemental Fig. 5].

Materials and methods

Protein purification and crystallization

Recombinant proteins were expressed using the Bac-to-Bac baculovirus expression system [Invitrogen]. In brief, the ORFs of RSPO1-2F, the LGR4-ECD, and the ZNRF3-ECD with an N-terminal gp67 signal peptide to facilitate secretion and a C-terminal 6-His tag were cloned into the pFastBac Dual vector [Invitrogen]. After transfection and virus amplification, the high-titer viruses were used to infect Sf9 cells, and the secreted proteins were purified from the medium using Ni-NiTA column and gel filtration chromatography. Baculoviruses encoding RSPO1-2F and the LGR4-ECD were concentrated into Sf9 cells to obtain the complex. The crystals were grown using sitting-drop vapor diffusion at 291 K by mixing equal volumes of protein and reservoir solution containing 0.1 M sodium citrate [pH 6.0] and 10% [v/v] PEG 6000.

Data collection and structural determination

Crystals were cryo-protected in reservoir solution supplemented with 20% [v/v] glycerol and flash-frozen in liquid nitrogen. Diffraction data were collected at the BL17U beam line of the Shanghai Synchrotron Research Facility (SSRF). Diffraction data were indexed, integrated, and scaled with the program HKL2000 [Otwinowski and Minor 1997]. The structure was determined by molecular replacement with PHASER [McCoy et al. 2007] and refined with PHENIX [Adams et al. 2002]. Structure validation was performed with PROCHECK [Laskowski et al. 1993]. More detailed procedures, including affinity measurements by SPR and STF reporter assays, are described in the Supplemental Material.

Protein Data Bank (PDB) deposition

The coordinates and diffraction data have been deposited in the PDB with accession code 4KT1. The coordinates and diffraction data have been deposited in the PDB with accession code 4KT1.

Acknowledgments

We thank J.W. Wang for assistance with structure determination, and J.H. He and other staff members at the Shanghai Synchrotron Research Facility (SSRF) beam line BL17U for help with data collection. We also thank Y.C. Chen for helpful discussion. This work was supported by the Ministry of Science and Technology (2010CB912402 and 2011CB910502), the Ministry of Health (2012ZX10001009), and the Fok Ying Tung Education Foundation to X.W., and the Ministry of Science and Technology (2011CB943803) to W.W.

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*Genes Dev.* 2013, 27: originally published online June 11, 2013
Access the most recent version at doi:10.1101/gad.219360.113

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**Supplemental Material**
http://genesdev.cshlp.org/content/suppl/2013/06/04/gad.219360.113.DC1

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