R-spondin1 Is a High Affinity Ligand for LRP6 and Induces LRP6 Phosphorylation and β-Catenin Signaling*

Qiou Wei‡, Chika Yokota†, Mikhail V. Semenov§, Brad Doble§, Jim Woodgett§, and Xi He‡‡

From the ‡Program of Neurobiology, Children’s Hospital Boston, Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115 and the §Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario MSG 1X5, Canada

R-spondin proteins are newly identified secreted molecules that activate β-catenin signaling. However, the mechanism of R-spondin action and its relationship with Wnt signaling remain unclear. Here we show that human R-spondin1 (hRspo1) is a high affinity ligand for the Wnt co-receptor LRP6 (Kd = 1.2 nM). hRspo1 induces glycogen synthase kinase 3-dependent phosphorylation and activation of LRP6. DKK1, an LRP6 antagonist, inhibits hRspo1-induced LRP6 phosphorylation. We further demonstrate that hRspo1 synergizes with Frizzled5 in Xenopus axis induction assays and induces the phosphorylation of Dishevelled, a cytoplasmic component downstream of Frizzled function. Our study reveals interesting similarity and distinction between Wnt and R-spondin signaling.

The Wnt/β-catenin signaling pathway plays pivotal roles in developmental processes and diseases (1). Wnt proteins are secreted and lipid-modified molecules (2). Depending on the cell type and receptor complement present, Wnt proteins can activate the “canonical” β-catenin-dependent signaling or β-catenin-independent signaling, including the Wnt/Ca2+ pathway and the planar cell polarity pathway (3). The canonical Wnt signaling operates via stabilization of the transcriptional co-activator β-catenin. In the absence of Wnt stimulation, cytosolic β-catenin is sequentially phosphorylated by casein kinase Iα and glycogen synthase kinase 3 (GSK3) in a complex composed of the scaffolding protein Axin and the tumor suppressor protein APC (4–6). Phosphorylated β-catenin is recognized by the F-box protein β-Trcp and degraded by the proteasome (7–9). Upon Wnt stimulation, β-catenin phosphorylation and degradation are inhibited, resulting in the accumulation of β-catenin, which translocates to the nucleus and interacts with the TCF/lymphoid enhancer factor family of transcription factors to activate Wnt target genes (10–13).

The extracellular Wnt ligand initiates signaling via its cell surface receptor complex, which consists of a member of the Frizzled (Fz) family of serpentine receptors and a co-receptor, low density lipoprotein receptor-related protein 5 or 6 (LRP5/6) (14–17). How Wnt activates Fz and LRP5/6 remains to be fully understood. It has been shown that LRP6 upon Wnt-induced phosphorylation binds to Axin, thereby enabling the Wnt receptor complex to directly regulate β-catenin phosphorylation, although the precise mechanism remains unclear (18–21).

In addition to Wnt family of proteins, other secreted molecules have been identified as ligands of Frizzled/LRP5/6 receptor complex and can either activate or inhibit the canonical Wnt/β-catenin signaling. For instance, Norrin activates Wnt/β-catenin signaling by specifically binding to Frizzled4 (22). On the other hand, Dickkopf-1, Wise, and SOST antagonize Wnt/β-catenin signaling through direct interaction with LRP5/6 (23–28). Spatially and temporally regulated expression of these molecules, together with the receptor complement in specific tissues, likely accomplishes the critical roles of Wnt/β-catenin signaling in both embryonic development and maintaining of homeostasis in adult tissues.

A novel family of secreted molecules, referred to as R-spondin (roof plate-specific spondin) (29, 30), has been identified that activate β-catenin signaling (31, 32). Four R-spondin proteins (Rspo1–4) exist in vertebrates, which do not bear sequence similarity to the Wnt family of proteins. Rspo1 was identified as a gene in mouse that is expressed in the developing spinal cord, and its expression requires Wnt activities (29). In Xenopus, Rspo2 was isolated in a functional cDNA expression screen for molecules that can activate a TCF/β-catenin-independent reporter expression in HEK293T cells (30). Like in mouse, Xenopus R-spondin genes are often co-expressed with and induced by Wnt genes during embryogenesis. Human Rspo1 (hRspo1) was identified by virtue of its ability to promote, upon overexpression in transgenic mice, robust proliferation of intestinal epithelial cells (31). hRspo1 was also found to be effective in preventing intestinal mucositis caused by cancer chemotherapy in a mouse model (31). This property of hRspo1 strongly correlates with its ability to induce β-catenin accumulation and activation of lymphoid enhancer factor/TCF target genes.

Despite the biological and potential therapeutic significance, the mechanism by which the R-spondin family of proteins activates β-catenin signaling is not fully understood. It has been proposed that the Frizzled-LRP5/6 complex may not act as R-spondin receptors, because of an apparently lack of detectable
**R-spondin Signaling through LRP6**

R-spondin2-Frizzled or R-spondin2-LRP5/6 interaction (30, 31). However, a recent study showed that R-spondin3 can be co-immunoprecipitated with the extracellular domain of either Frizzled8 or LRP6 (32). In an effort to search for receptor(s) involved in R-spondin signaling, we found that LRP6, but not Frizzled, is a high affinity receptor for hRspo1 ($K_d = 1.2$ nm). In both mammalian cells and *Xenopus* embryo injection, LRP6 strongly synergizes with hRspo1 to stimulate β-catenin signaling. Furthermore, hRspo1 induces LRP6 phosphorylation, which is a key event involved in the activation of Wnt/β-catenin signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**HEK293T cells and MEF cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum at standard conditions. L cells and COS7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% newborn calf serum. For transient transfection, the TOPFLASH reporter and pRL-TK constructs were used. Cells were cultured in 24-well plates, and transfection was performed using Lipofectamine-2000 according to the manufacturer’s suggested protocol. Dual luciferase assays were carried out 48 h after transfection according to manufacturer’s instruction (Promega). To knock down GSK3β protein was sterilized through a 0.22-μm filter and stored at −80 °C.

**DNA Constructs—**Human R-spondin1 full-length cDNA was amplified from human brain tissue by RT-PCR using primers as reported (30). The amplified gene was confirmed by sequencing and was cloned into pSecAP-MH vector for expression in mammalian cells. It was also cloned into pCS2+ vector for in vitro transcription to generate mRNA used in *Xenopus* injection. Plasmids encoding Dkk-1, mFz8CRD-IgG, IgG, LRP6N-IgG, Frizzled, and LRP6 were used as described previously (15, 24, 33). Coding regions of the first plus the second propellers (ECD12) or the third plus the fourth propellers (ECD34) of the LRP6 extracellular domain were amplified by PCR and cloned upstream in-frame of the human IgG Fc fragment in pCDNA3.1. These plasmids express fusion proteins as ECD12-IgG, ECD34-IgG, or ECD12-IgG with ECD34-IgG. The purity of the final hRspo1 protein was at least 99% as evaluated by Coomassie gel staining. Purified protein was sterilized through a 0.22-μm filter and stored at −80 °C.

**Alkaline Phosphatase Staining and Binding Affinity Assay—**Cell surface binding under native conditions was performed as described (34). Briefly, COS7 cells were incubated at 4 °C for 2 h with AP conditioned medium or with hRspo1-AP conditioned medium. After incubation, cells were washed six times with washing buffer (0.5 mg/ml bovine serum albumin in Hanks’ balanced salt solution, the fixed cells were heat-inactivated at 65 °C overnight. Cells were stained at room temperature with bromochloroindolyl phosphate (0.17 mg/ml) and nitro blue tetrazolium (0.33 mg/ml) until color developed. Staining and fluorescence images were taken using an anatomic microscope (Leica). Solid phase binding affinity measurement was performed as described (24).

**Xenopus laevis Embryo Manipulations and RT-PCR—**Procedures for embryo staging, injection, and animal cap assays were done as described (35). Total RNA isolation and RT-PCR were performed as described (35). EF1α was used as positive control. Reverse transcriptase negative (RT−) reactions showed no evidence of genomic DNA contamination. The primers used were as described previously (36).

**RESULTS**

**Activation of Wnt/β-Catenin Signaling Pathway by Purified Recombinant hRspo1—**We generated expression vectors for various versions of tagged hRspo1 or truncated hRspo1 (Fig. 1A). These plasmids were transiently transfected into HEK293T cells, and CM were collected 2 days after transfec-
hRspo1 CM or Wnt3a CM were not able to stabilize total cell lysates were used for Western blotting, and (30, 32). However, hRspo1 and Wnt3a synergized to stabilize stabilizing cytosolic Nin signaling in mouse L cells. hRspo1 alone was not capable of

We fractionated hRspo1 CM sequentially through heparin affinity columns, which resulted in highly purified hRspo1 with 95% recovery from the CM and more than 1000-fold enrichment (Fig. 1C and data not shown). As shown in Fig. 1D, purified hRspo1 alone was sufficient to stabilize cytosolic β-catenin, and it also strongly synergized with Wnt3a CM to induce accumulation of β-catenin in HEK293T cells.

hRspo1 Synergizes with LRP6 to Stimulate Wnt/β-Catenin Signaling in Mammalian Cells—Previous studies suggested that Xenopus Rspo2 and mouse Rspo3 have the ability to activate TCF/β-catenin-dependent transcription (30, 32). To further characterize hRspo1-induced signaling, transient transfection experiments were performed in HEK293T cells using a TCF/β-catenin reporter (TOPFLASH). hRspo1 alone induced TOPFLASH luciferase activity, likely via using the endogenous receptor(s); hRspo1 and LRP6 synergized strongly when co-transfected (Fig. 2A). When hRspo1 was co-transfected with different HA-Frizzled cDNAs, it synergized with Frizzled5 and -8 and, to a lesser extent, with Frizzled1 and -4 to stimulate TOPFLASH reporter activity (Fig. 2B). Expression of hRspo1 with Frizzled2, -3, -6, -7, or -9 did not further stimulate TOPFLASH luciferase reporter activity (Fig. 2B and data not shown). The expression level of different HA-Frizzled proteins was comparable as indicated by Western blotting (Fig. 2C). These data suggest that LRP6 and a few selected Frizzled proteins participate in hRspo1 signaling.

**Cell Surface Binding of hRspo1-AP to LRP6**—As shown in Fig. 1B, hRspo1-alkaline phosphatase fusion protein (hRspo1-AP) was efficiently secreted and was capable of stimulating β-catenin signaling the same extent as the wild type hRspo1. When hRspo1-AP CM was incubated at room temperature with HEK293T cells for 2 h and visualized subsequently with bromochloroindolyl phosphate/nitro blue tetrazolium histochemical reaction, strong cell-associated AP activity was observed (data not shown). This was also true for other cell types tested, including L cells, MEFs, and COS7 cells (Fig. 3A). As a control, the secreted AP protein did not stain any of these cells under the same conditions (Fig. 3A and data not shown). The hRspo1-AP staining could be due to binding of hRspo1-AP to the extracellular matrix, similar to that of Wingless, which adheres to the extracellular matrix of a variety of cultured cells (37), or it could be due to the binding of hRspo1-AP to endogenously expressed receptor(s) in these cells.

We used COS7 cells to test hRspo1-AP binding to candidate receptors because these cells have been successfully used for

**Figure 1. Characterization of tagged hRspo1 variants and purification of biologically active hRspo1 from CM.** A, schematic representation of various tagged hRspo1 and mutants. SP, signal peptide; FU, furin repeats; TSP, thrombospondin domain; CT, carboxyl terminus. B, CM containing indicated hRspo1 proteins, in conjunction with suboptimal Wnt3a CM, stabilizes β-catenin in mouse L cells. After 6 h of CM treatment, total cell lysates were used for Western blotting, and β-actin was used as a loading control. C, Coomassie staining of purified hRspo1 by a tandem affinity protocol. The arrow indicates purified hRspo1. Lane M, mass marker. D, purified hRspo1 stabilizes β-catenin in HEK293T cells. HEK293T cells were treated for 2 h with purified hRspo1 with or without Wnt3a CM as indicated. Cytosolic extracts were used to blot for β-catenin. β-Actin was used as a loading control.
ligand-receptor binding studies (34). We performed the binding assay at 4 °C to reduce the possible internalization and degradation of ligand-receptor complexes. As shown in Fig. 3A, hRspo1-AP showed significantly stronger binding to cells transfected with LRP6 than to the untransfected cells, and this binding correlated with the expression of green fluorescence protein which marked the transfected cells (Fig. 3B). In contrast, cells expressing Frizzled1, -2, -5, and -8 did not show significant binding above the control level (Fig. 3A and data not shown). This was surprising given that Frizzled1, -5, or -8 can synergize with hRspo1 in signaling (Fig. 2B). These data suggest that LRP6 is a specific binding receptor for hRspo1.

To determine the binding affinity of hRspo1 to LRP6, we performed a solid phase enzyme-linked binding assay in which increasing amounts of hRspo1-AP were incubated with immobilized IgG, mFz8CRD-IgG (mouse Frizzled8 cysteine-rich domain fused with IgG) (33), or LRP6N-IgG (LRP6 extracellular domain fused with IgG) (15). As shown in Fig. 3C, hRspo1-AP bound to LRP6N-IgG, and the binding was saturable. In contrast, hRspo1-AP did not bind to IgG alone. hRspo1-AP did not bind to mFz8CRD-IgG, or bound with very low affinity, consistent with the lacking of detectable hRspo1-AP-binding to Frizzled transfected cells (Fig. 3A). A Scatchard plot derived from the hRspo1-AP/LRP6N-IgG binding data suggested a binding affinity (Kd) at 1.2 nM (Fig. 3D). Therefore, LRP6 is a high affinity receptor for hRspo1.

To further map the interaction between hRspo1 and LRP6, we made constructs expressing secreted forms of the extracellular propeller domains of LRP6, propellers 1 plus 2 (ECD12-IgG), and propellers 3 plus 4 (ECD34-IgG), which are fused with the human IgG Fc fragment. After transfection into HEK293T cells, these fusion proteins, similar to LRP6N-IgG, were secreted into CM as indicated by Western blotting (Fig. 4A). Co-immunoprecipitation was performed using hRspo1-MycHis CM and CM containing IgG, LRP6N-IgG, ECD12-IgG, or ECD34-IgG. As shown in Fig. 4B, hRspo1 was co-immunoprecipitated with LRP6N-IgG, ECD12-IgG, or ECD34-IgG but not with IgG alone. hRspo1 binding to LRP6N-IgG appeared to be stronger than to either ECD12-IgG or ECD34-IgG alone, as significantly more abundant ECD12-IgG or ECD34-IgG in CM co-precipitated comparable amounts of hRspo1 as LRP6N-IgG did (Fig. 4B). These data suggest that hRspo1 may interact with multiple propeller repeat domains.

hRspo1 Induces LRP6 Phosphorylation—Our previous studies have shown that phosphorylation of LRP6 is critical for signaling induced by the Wnt family of proteins. A PPPSPXS motif, which is reiterated five times in the LRP6 intracellular domain, is sequentially phosphorylated by GSK3 and casein kinase 1 upon Wnt induction (18, 19). Phosphorylation of this motif promotes the engagement of LRP6 with the scaffolding protein Axin, thereby transducing the extracellular Wnt signal into intracellular compartments (18–20). To investigate whether this mechanism is also involved in R-spondin-mediated signaling, we treated MEFs with purified hRspo1. As shown in Fig. 5A, phosphorylation of the endogenous LRP6 was induced within 15 min of exposure to 400 ng/ml hRspo1, as detected by a phosphorylated PPPSPXS motif-specific antibody (18). The peak level of phosphorylation appeared to be between 2 and 8 h after hRspo1 stimulation, a temporal pattern that was similar to Wnt3a-induced LRP6 phosphorylation in these cells (data not shown). hRspo1 induced LRP6 phosphorylation in a dose-dependent manner to about 800 ng/ml; higher doses of

![Figure 2](https://example.com/figure2.png)

**FIGURE 2.** hRspo1 and LRP6 synergistically stimulate a TCF/β-catenin luciferase reporter. A, HEK293T cells were transfected with expression plasmids as indicated with a combination of TOPFLASH and Renilla-TK reporter gene constructs. Relative luciferase ratio (Firefly/Renilla) was used to obtain values of fold induction compared with a vector only control. Data are the mean ± S.E. from at least three replicates. B, transfection experiments in HEK293T cells as in A, but using HA-tagged Frizzled expression plasmids. Statistical analysis was performed by Student’s t test compared with hRspo1 alone (*, p < 0.05). C, cell lysates from B were used to blot for HA-Frizzled and hRspo1-MycHis. The nonspecific band in anti-HA blot is indicated with an asterisk.
hRspo1 (above 1,000 ng/ml) did not further increase the levels of LRP6 phosphorylation (Fig. 5B).

We have demonstrated that purified hRspo1 strongly synergizes with Wnt3a to induce the accumulation of β-catenin (Fig. 1D). To investigate whether this is correlated with LRP6 phosphorylation levels, MEFs were treated with Wnt3a CM with or without hRspo1. As shown in Fig. 5C, Wnt3a alone induced phosphorylation of the endogenous LRP6 in a time-dependent manner. In the presence of 400 ng/ml hRspo1, a more rapid induction of LRP6 phosphorylation was observed. To further confirm this result, we reduced the amount of Wnt3a CM to a suboptimal level that barely induced LRP6 phosphorylation on its own; however, the combination of Wnt3a with purified hRspo1 resulted in a significant increase of LRP6 phosphorylation compared with treatment of either Wnt3a or hRspo1 alone (Fig. 5D). These data suggest that purified hRspo1 is capable of inducing LRP6 phosphorylation and has the ability to synergize with Wnt3a to induce LRP6 phosphorylation.

Our previous study indicates that Wnt-induced phosphorylation of the LRP6 PPPSP motif is mediated by GSK3 (19). Thus we asked if GSK3 is responsible for hRspo1-induced phosphorylation of LRP6. GSK3β−/−;GSK3αfloxp/fox MEFs were treated with Wnt3a CM and/or purified hRspo1. Treatment of Wnt3a CM or purified hRspo1 caused a significant increase in the phosphorylation level of the PPPSP motif in these cells (Fig. 6A). However, when GSK3α was knocked down by the expression of the Cre recombinase, LRP6 phosphorylation on the PPPSP motif was significantly reduced (Fig. 6A). The residual LRP6 phosphorylation was roughly correlated with the residual GSK3α level, which may be due to incomplete deletion of the Gsk3α gene by Cre and/or long half-life of GSK3α mRNA/protein (Fig. 6A). These data indicate that, like Wnt family of proteins, hRspo1-induced LRP6 phosphorylation is also mediated by GSK3.

Wnt3a/hRspo1-induced Phosphorylation of LRP6 Is Inhibited by DKK1—DKK1 is a high affinity ligand of LRP6 (Kd = 0.5 nm), and binding of DKK1 to LRP6 antagonizes Wnt/β-catenin signaling (24, 26, 27). To test whether DKK1 has any effect on hRspo1-induced LRP6 phosphorylation, MEFs were treated...
R-spondin Signaling through LRP6

A.

| Time (hr) | 0 | 0.25 | 0.5 | 1 | 2 | 4 | 6 | 8 | 10 |
|-----------|---|------|-----|---|---|----|---|---|----|
| pLRP6     | - | -    | -   | + | + | +  | + | + | +  |
| β-Actin   | - | -    | -   | - | - | +  | + | + | +  |

B.

| hRspo1-MycHis (ng/ml) | 0 | 50 | 100 | 500 | 1000 | 1500 | 2000 | 2500 |
|-----------------------|---|-----|------|------|-------|-------|-------|-------|
| pLRP6                 | - | -   | -    | -    | +     | +     | +     | +     |
| β-Actin               | - | -   | -    | -    | -     | -     | -     | -     |

C.

| Time (hr) | 0 | 0.25 | 0.5 | 1 | 2 |
|-----------|---|------|-----|---|---|
| pLRP6     | - | -    | +   | + | + |
| β-Actin   | - | -    | -   | + | + |

FIGURE 5. hRspo1 induced GSK3-dependent phosphorylation of the endogenous LRP6 in MEFs. A, time-dependent induction of LRP6 phosphorylation by hRspo1. MEF cells were treated with 400 ng/ml of purified hRspo1. Total cell lysates were used to blot for phosphorylated LRP6 with a phosphorylated PPPSP motif-specific antibody. B, concentration-dependent induction of LRP6 phosphorylation by hRspo1. MEF cells were treated with the indicated dose of hRspo1 for 2 h. C and D, hRspo1 and Wnt3a synergistically induced phosphorylation of LRP6 in MEF cells. C, 400 ng/ml of hRspo1 or 100% of Wnt3a conditioned media were used. D, MEF cells were treated by indicated doses of hRspo1 and/or Wnt3a CM for 2 h.

R-spondin Signaling through LRP6—Our data suggested that hRspo1 can functionally engage specific Frizzled receptors. Thus we tested whether hRspo1 is capable of inducing Dishevelled phosphorylation, which is a signature for Frizzled activation (42–44). As shown in Fig. 8, treatment of HEK293T cells with Wnt3a CM induced an upper shift of the Dvl3 protein. Treatment of samples with calf intestinal phosphatase caused the disappearance of the shifted band and increasing intensity of the lower band, confirming the upper band was the phosphorylated form of Dvl3 (Ref. 42 and data not shown). Purified hRspo1 also induced Dvl3 phosphorylation in a dose-dependent manner (Fig. 8).

DISCUSSION

R-spondin proteins are novel secreted signaling molecules that are expressed in temporal and tissue-specific patterns during vertebrate development (29, 30, 45). Xenopus Rspo2 is involved in embryonic myogenesis (30), whereas in transgenic mice, hRspo1 is a potent and specific mitogen for gastrointestinal epithelia and exhibits therapeutic potential in the preven-
tion of gastrointestinal mucositis caused by cancer chemotherapy (31). Both in tissue culture and in vivo, R-spondin proteins have been shown to have the ability to stabilize cytosolic β-catenin and thus to activate β-catenin signaling (30, 31, 46). However, the mechanism by which R-spondin proteins activate the β-catenin signaling pathway is not well understood. In this study, we demonstrate that hRspo1 is a high affinity ligand for the Wnt co-receptor LRP6 and induces LRP6 phosphorylation/activation. We also show that hRspo1 has a strong functional interaction with specific Frizzled, in particular Frizzled5, and induces the phosphorylation of Dishevelled, a Frizzled downstream signaling component. Our study therefore reveals some common themes and interesting differences between R-spondin and Wnt signaling.

**hRspo1 Protein**—R-spondin proteins contain an amino-terminal signal peptide, a cysteine-rich region that is composed of two furin repeats, a thrombospondin-type domain, and a carboxyl terminus rich in positively charged residues (Fig. 1A). Our results demonstrate that, like Xenopus Rspo2 and murine Rspo3 (32), the furin repeat domain, but neither the thrombospondin domain nor the carboxyl terminus, is required for the activation of β-catenin signaling (Fig. 1). Unlike Xenopus Rspo2 and murine Rspo1 (29, 30), which are poorly secreted when expressed in mammalian cells, we and Kim et al. (31) have found that hRspo1 is efficiently secreted upon overexpression in HEK293T cells. Using RT-PCR, we also found that hRspo1 is endogenously expressed in HEK293T cells (data not shown). Distinct from Wnt proteins, which are lipid-modified and are found in the detergent phase (2), we found that hRspo1 is hydrophilic and mainly distributed in the water-soluble phase (data not shown) and was in fact purified in the absence of any detergent (Fig. 1C and "Experimental Procedures"). Based on these findings, we have developed a tandem affinity purification protocol and isolated a biologically active hRspo1 (Fig. 1D).

**LRP6 Is a High Affinity Receptor for hRspo1**—Previous efforts to investigate the mechanism by which R-spondin activates β-catenin signaling have yielded conflicting results. In one report, attempts to establish binding between Xenopus Rspo2 with LR6 or Frizzled5 proteins were unsuccessful (30). A recent study, however, showed co-immunoprecipitation of murine Rspo3 with the extracellular domain of either LRP6 or Frizzled8 (32). Our data provide definitive evidence that LR6 is a critical binding and functional receptor for hRspo1. First, hRspo1 binds

![Figure 7](image-url)  
**FIGURE 7. hRspo1 induces axis duplication and Xnr3 expression in the presence of LRP6 or Frizzled5 in Xenopus.** A, hRspo1 induces axis duplication when co-injected with LR6 or Frizzled5. mRNA was injected into the ventral marginal two cells at the 4–8-cell stage. The arrowhead indicates the location of the secondary axis. Note the complete axis duplication in the case of hRspo1 plus Frizzled5. B, summary of data from co-injection experiments. The white bar indicates the percentage of injected embryos that had trunk-tail type of secondary structures. The black bar indicates the percentage of injected embryos that had the secondary head structures. n, number of embryos injected/examined. un inj indicates not injected. C, Xnr3 was induced synergistically in the animal caps by co-injection of hRspo1 and LR6 or Frizzled5. mRNA was injected into the animal pole of two-cell stage embryos, and the animal caps were dissected at stage 9.5 and cultured until stage 10.5 for RT-PCR analysis. PCR of samples without reverse transcription (RT−) was used as negative control showing no contamination of genomic DNA. The whole embryo (WE) was used as a positive control for RT-PCR. RT-PCR of EF-1α was used as a loading control.

![Figure 8](image-url)  
**FIGURE 8. hRspo1 induced phosphorylation of Dishevelled in HEK293T cells.** HEK293T cells were treated with 50% (+) or 100% (+) of Wnt3a CM or 200–800 ng/ml of purified hRspo1. After a 2-h treatment, cells were harvested, and total cell lysates were used to blot for Dvl3. Phosphorylated Dvl3 is indicated with an asterisk.
R-spondin Signaling through LRP6

to cells that are transfected with LRP6; second, we have measured the binding affinity \( (K_d) \) between hRspo1 and LRP6 to be at 1.2 nM (Fig. 3); third, hRspo1 induces LRP6 phosphorylation at the PPPSP motif (Fig. 5), a hallmark of LRP6 activation (18); and fourth, hRspo1-induced LRP6 phosphorylation is blocked by the LRP6 antagonist DKK1 (Fig. 6B). These results, together with the functional synergy observed between R-spondin proteins and LRP6 in this and previous studies (Figs. 2 and 7) (30, 32), argue unambiguously for a ligand-receptor relationship between R-spondin proteins and LRP6. Our mapping study further suggests that hRspo1 can bind to at least two separate LRP6 extracellular domains that are each composed of the \( \beta \)-propeller repeats.

Relationship between R-spondin and Frizzled Proteins—Our data show that hRspo1 binds to LRP6 with high affinity but binds poorly, if at all, to Frizzled receptors on the cell surface and in vitro (Figs. 3 and 4). These findings are in line with a recent study showing that the extracellular domain of LRP6 co-immunoprecipitates R-spondin proteins much more effectively than that of Frizzled8 (32). In addition, co-expression of LRP6 with Frizzled5 or Frizzled8 in COS7 cells does not appear to confer more hRspo1 binding than LRP6 alone (data not shown), suggesting that Frizzled5 or Frizzled8 binding to hRspo1 remains minimal even in the presence of overexpressed LRP6. However, we observed functional interactions between R-spondin and Frizzled proteins, particularly Frizzled5 and Frizzled8, in the activation of TCF/\( \beta \)-catenin reporters in mammalian cells (Fig. 2B) (30). In Xenopus embryo injection, hRspo1 and Frizzled5 (but not other Frizzled receptors, including Frizzled8) synergistically activate \( \beta \)-catenin signaling during axis induction (Fig. 7). The molecular basis underlying the hRspo1-Frizzled functional interaction and the difference between mammalian cells and Xenopus embryos is at the moment unknown. Further evidence suggestive of a Rspo-Frizzled relationship is our observation that hRspo1 induces Dishevelled3 phosphorylation, which is often indicative of Frizzled activation. Possibilities to reconcile these seemingly disparate binding and functional results include the following: (i) a weak but significant Rspo-Frizzled binding (Fig. 3C) that is beyond our detection method; and (ii) the existence of additional cofactor(s) or co-receptor(s), which may be differentially expressed in embryos and in mammalian cells and may help to bridge R-spondin and Frizzled proteins.

Comparisons between R-spondin and Wnt Signaling—Our data, in conjunction with previous studies (31, 32), allow us to make mechanistic comparisons between R-spondin and Wnt signaling. Either R-spondin or Wnt is able to do the following: (i) stabilize \( \beta \)-catenin and activate TCF/\( \beta \)-catenin-dependent transcriptional response; (ii) utilize LRP6 as a binding and signaling receptor; (iii) induce LRP6 phosphorylation/activation via GSK3; (iv) be antagonized by LRP6-specific inhibitor DKK1; (v) can functionally couple to a specific Frizzled receptor or receptors; and (vi) can induce Dishevelled phosphorylation. These features suggest a similar mechanism for R-spondin and Wnt signaling. In addition, hRspo1 induction of Dishevelled phosphorylation raises the possibility whether R-spondin can also activate noncanonical (\( \beta \)-catenin-independent) Wnt/Frizzled pathways.

However, there are important distinctions between R-spondin and Wnt actions, particularly with regard to the receptor preference. Although Wnt binds to Frizzled receptors with high affinity (with \( K_d \) at about 1 to 10 nM range) (33, 47), Wnt binding to LRP6 is significantly weaker (reviewed in (17) and in some cases difficult to detect (48). By contrast, R-spondin binds to LRP6 with high affinity but binds to Frizzled to a significantly less extent (32) or poorly (this study). Future studies will be required to address the Rspo-Frizzled relationship. We found that hRspo1 activity appears to be not as potent as that of Wnt3a CM in inducing LRP6 phosphorylation and \( \beta \)-catenin stabilization, but further effort with additional purified R-spondin and Wnt proteins is needed to make informative comparisons. It is also worth noting that hRspo1 and suboptimal amounts of Wnt3a show strong synergy in LRP6 phosphorylation and \( \beta \)-catenin stabilization (Figs. 1B and 5D). In both Xenopus and mouse embryos, expression patterns of different R-spondin genes often overlap with and in fact depend on the expression of Wnt genes (29, 30). Thus R-spondin proteins may represent positive feedback that reinforces Wnt signaling during development.

Acknowledgment—We thank members of the He laboratory for comments and suggestions.

REFERENCES

1. Nusse, R. (2005) Cell Res. 15, 28–32
2. Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R., Illi, and Nusse, R. (2003) Nature 423, 448–452
3. Strutt, D. (2003) Development (Camb.) 130, 4501–4513
4. Ikeda, S., Kishida, S., Yamamoto, H., Murali, H., Koyama, S., and Kikuchi, A. (1998) EMBO J. 17, 1371–1384
5. Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G. H., Tan, Y., Zhang, Z., Lin, X., and He, X. (2002) Cell 108, 837–847
6. Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S., and Kikuchi, A. (1998) J. Biol. Chem. 273, 10823–10826
7. Liu, C., Kato, Y., Zhang, Z., Do, V. M., Yankner, B. A., and He, X. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6273–6278
8. Behrens, J., Jerchow, B. A., Wurtele, M., Grimm, J., Ashbrand, C., Wirtz, R., Kuhl, M., Wedlich, D., and Birchmeier, W. (1998) Science 280, 596–599
9. Salic, A., Lee, E., Mayer, L., and Kirschner, M. W. (2000) Mol. Cell 5, 523–532
10. Hsu, S. C., Galceran, J., and Grosschedl, R. (1998) Mol. Cell. Biol. 18, 4807–4818
11. Tolwinski, N. S., and Wieschaus, E. (2004) Plos Biol. 2, E95
12. Willert, K., Shibamoto, S., and Nusse, R. (1999) Genes Dev. 13, 1768–1773
13. Bauer, A., Chauvet, S., Huber, O., Usseglio, F., Rothbacher, U., Aragón, D., Klemmer, R., and Pradel, J. (2000) EMBO J. 19, 6121–6130
14. Pinson, K. L., Brennan, J., Monksly, S., Avery, B. J., and Skarnes, W. C. (2000) Nature 407, 535–538
15. Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P., and He, X. (2000) Nature 407, 530–535
16. Wehrli, M., Dougan, S. T., Caldwell, K., O’Keefe, L., Schwartz, S., Vaizel-Sakamoto, I., Koyama, S., and Kikuchi, A. (1998) J. Biol. Chem. 273, 10823–10826
17. Hsu, S. C., Galceran, J., and Grosschedl, R. (1998) Mol. Cell. Biol. 18, 4807–4818
18. Tamai, K., Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J., and He, X. (2005) Nature 438, 873–877
19. Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J., and He, X. (2005) Nature 438, 873–877
20. Mao, J., Wang, J., Liu, B., Pan, W., Farr, G. H., III, Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L., and Wu, D. (2001) Mol. Cell 7, 801–809
21. Tolwinski, N. S., Wehrli, M., Rives, A., Erdeniz, N., DiNardo, S., and Wieschaus, E. (2003) Dev. Cell 4, 407–418
22. Xu, Q., Wang, Y., Dabdoub, A., Smallwood, P. M., Williams, J., Woods, C., Kelley, M. W., Jiang, L., Tasman, W., Zhang, K., and Nathans, J. (2004) Cell 116, 883–895
23. Li, X., Zhang, Y., Liu, W., Liu, P., Zhang, J., Harris, S. E., and Wu, D. (2005) J. Biol. Chem. 280, 19883–19887
24. Semenov, M. V., Tamai, K., Brott, B. K., Kuhl, M., Sokol, S., and He, X. (2001) Curr. Biol. 11, 951–961
25. Semenov, M., Tamai, K., and He, X. (2005) J. Biol. Chem. 280, 26770–26775
26. Bafico, A., Liu, G., Yaniv, A., Gazit, A., and Aaronson, S. A. (2001) Nat. Cell Biol. 3, 683–686
27. Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A., and Niehrs, C. (2001) Nature 411, 321–325
28. Itasaki, N., Jones, C. M., Mercurio, S., Rowe, A., Domingos, P. M., Smith, J. C., and Kranz, R. (2003) Develop. Camb. 130, 4295–4305
29. Kamata, T., Katsube, K., Michikawa, M., Yamada, M., Takada, S., and Mizusawa, H. (2004) Biochem. Biophys. Acta 1676, 51–62
30. Kazanskaya, O., Glinka, A., del Barco Barrantes, I., Stannek, P., Niehrs, C., and Wu, W. (2004) Dev. Cell 7, 525–534
31. Kim, K. A., Katriti, M., Zhao, J., Oshima, T., Tang, T., Binnerts, M., Liu, Y., Boyle, B., Park, E., Emte, P., Funk, W. D., and Tomizuka, K. (2005) Science 309, 1256–1259
32. Nam, J. I., Turcotte, T. J., Smith, P. F., Choi, S., and Yoon, J. K. (2006) J. Biol. Chem. 281, 13247–13257
33. Hsieh, J. C., Rattner, A., Smallwood, P. M., and Nathans, J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3546–3551
34. Flanagan, J. G., Cheng, H. J., Feldheim, D. A., Hattori, M., Lu, Q., and Vanderhaeghen, P. (2000) Methods Enzymol. 327, 19–35
35. Kato, Y., Shi, Y., and He, X. (1999) J. Neurosci. 19, 9364–9373
36. Darras, S., Marikawa, Y., Elinson, R. P., and Lemaire, P. (1997) Develop. Camb. 124, 4275–4286
37. Bhonot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, I., and Nusse, R. (1996) Nature 382, 225–230
38. Zhang, Y., Wang, Y., Li, X., Zhang, J., Mao, J., Li, Z., Zheng, J., Li, L., Harris, S., and Wu, D. (2004) Mol. Cell. Biol. 24, 4677–4684
39. Mao, B., Wu, W., Davidson, G., Marhold, J., Li, M., Mecler, B. M., Delius, H., Hoppe, D., Stannek, P., Walter, C., Glinka, A., and Niehrs, C. (2002) Nature 417, 664–667
40. Funayama, N., Fagotto, F., McCrea, P., and Gumbiner, B. M. (1995) J. Cell Biol. 128, 959–968
41. McKendry, R., Hsu, S. C., Harland, R. M., and Grosschedl, R. (1997) Dev. Biol. 192, 420–431
42. Lee, J. S., Ishimoto, A., and Yanagawa, S. (1999) J. Biol. Chem. 274, 21464–21470
43. Rothbacher, U., Laurent, M. N., Deardorff, M. A., Klein, P. S., Cho, K. W., and Fraser, S. E. (2000) EMBO J. 19, 1010–1022
44. Gonzalez-Sancho, J. M., Brennan, K. R., Castelo-Soccio, L. A., and Brown, A. M. (2004) Mol. Cell. Biol. 24, 4757–4768
45. Chen, Y., Wang, S., Tang, R., Yang, Q. S., Zhao, E., Chao, Y., Ying, K., Xie, Y., and Mao, Y. M. (2002) Mol. Biol. Rep. 29, 287–292
46. Kim, K. A., Zhao, J., Andarmani, S., Katriti, M., Oshima, T., Binnerts, M. E., Abo, A., Tomizuka, K., and Funk, W. D. (2006) Cell Cycle 5, 23–26
47. Rulifson, E. J., Wu, C. H., and Nusse, R. (2000) Mol. Cell 6, 117–126
48. Wu, C. H., and Nusse, R. (2000) J. Biol. Chem. 277, 41762–41769