Interaction between LRP5 and Frat1 Mediates the Activation of the Wnt Canonical Pathway*

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Low density lipoprotein receptor-related protein 5 (LRP5) has been identified as a Wnt co-receptor involved in the activation of the β-catenin signaling pathway. To improve our understanding of the molecular mechanisms by which LRP5 triggers the canonical Wnt signaling cascade, we have screened for potential partners of LRP5 using the yeast two-hybrid system and identified Frat1 as a protein interacting with the cytoplasmic domain of LRP5. We demonstrate here that LRP5/Frat1 interaction is involved in β-catenin nuclear translocation and TCF-1 transcriptional activation. The addition of Wnt3a or overexpression of constitutively active truncated LRP5 (LRP5C) induces Frat1 recruitment to the cell membrane. Overexpression of a dominant negative form of disheveled (Dvl) shows that this protein positively affects LRP5/Frat1 interaction. Furthermore, the fact that dominant negative Dvl does not interfere with LRP5C/Frat1 interaction can explain how LRP5C is capable of acting independently of this major Wnt signaling player. Axin, which has been shown to interact with LRP5 and to be recruited to the membrane through this interaction, was found to co-immunoprecipitate with Frat1 and LRP5. We propose that recruitment of Axin and Frat1 to the membrane by LRP5 leads to both Axin degradation and Frat1-mediated inhibition of glycogen synthase kinase-3. As a consequence, β-catenin is no longer bound to Axin or phosphorylated by glycogen synthase kinase-3, resulting in TCF-1 activation.

Wnts are cysteine-rich secreted proteins involved in a wide range of developmental processes such as embryonic axis specification and organogenesis (1). Wnts appear to activate a variety of signaling pathways both in vertebrates and in invertebrates. In the so-called “canonical” Wnt/β-catenin pathway, the interaction between Wnt and frizzled receptors leads to inactivation of the kinase GSK-3β. Genetic epistasis experiments suggest that disheveled lies upstream and represses the activity of GSK-3β. As a consequence, β-catenin is stabilized in the cytoplasm and then forms a complex with TCF/LEF to activate transcription of target genes. Frizzled proteins have been shown to function as Wnt receptors (2), and they constitute a large family of seven transmembrane receptors with at least 10 members in mammals (2, 3). All frizzled receptors have a conserved extracellular cysteine-rich domain followed by seven putative transmembrane segments. Their cytoplasmic regions differ in length and sequence. Functional analyses in Drosophila and Xenopus embryos indicate that frizzled proteins have distinct functions in Wnt/β-catenin signaling (4). More recently, members of the low density lipoprotein receptor-related protein family (LRP) have been shown to be co-receptors for Wnt ligands (5–9). The two closely related proteins LRP5 and LRP6 are single-pass transmembrane receptors that associate with frizzled receptors in a Wnt-dependent manner. There is an increasing body of evidence showing that LRP5/6 are required for Wnt/β-catenin signaling. Beside Wnts, dickkopf proteins (Dkk), mainly Dkk1, Dkk2, and Dkk4, were also shown to bind with high affinity to LRP5/6 and to inhibit Wnt canonical signaling (6, 10–12). Despite the fact that modulating LRP5/6 receptor activity by extracellular ligands like Wnts or Dkks leads respectively to the activation or inhibition of β-catenin signaling pathway, it is unclear how these receptors triggers the signaling cascade in the intracellular compartment. Although it has been previously demonstrated that LRP5/6 interacts with Axin (5), this interaction only in part explains the mechanism by which this receptor regulates the β-catenin pathway.

In the present report we have used the intracellular cytoplasmic domain of LRP5 to screen for potential partners using the yeast two-hybrid system. Our screen led us to the identification of Frat1 as an LRP5-interacting protein. We demonstrate that LRP5 and Frat1 interact in cells and cooperate to induce β-catenin signaling. In addition, we present data suggesting that Frat1 activity is dependent on LRP5 expression.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The Matchmaker yeast two-hybrid (Y2H) system and the mouse 11- and 19-day embryo cDNA libraries were purchased from Clontech. The bait for library screening was the intracellular domain of LRP5 (1419–1615 residues). Y2H screening was carried out as suggested by the manufacturer. The interaction of the target proteins was determined phenotypically by growing of yeast clones on His-deficient medium and by measuring β-galactosidase activity. For a first confirmation of interaction with target proteins both bait and prey constructs were retrieved from the yeast clone of interest (as described by the manufacturer), reintroduced again in the same yeast strain, and assayed for their ability to grow on His-deficient medium and to induce β-galactosidase activity. To further confirm interactions, bait and prey cDNAs were removed from their original plasmids, recloned using appropriate restriction sites in prey and bait plasmids, respectively, reintroduced in the same yeast strain, and confirmed for their ability to grow on His-deficient medium and to induce β-galactosidase activity.

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The abbreviations used are: GSK-3β, glycogen synthase kinase 3β; LRP, low density lipoprotein receptor-related protein; Y2H, yeast two-hybrid; Wnt3a-CM, Wnt3a-conditioned medium; IP, immunoprecipitation; GFP, green fluorescent protein.
**Frat1 Interacts with LRPS**

Wnt3a-conditioned Medium Preparation—Wnt3a-conditioned medium (Wnt3a-CM) was prepared as described by Shibamoto et al. (13). Briefly, Wnt3a-producing L cells were seeded at a density of 6 × 10^6 cells/cm^2 in a 125-cm^2 flask containing Dulbecco's modified Eagle's medium with 10% fetal calf serum. 24 h after this, the medium was changed to Dulbecco's modified Eagle's medium with 2% fetal calf serum, and cells were cultured for 3 days. After harvesting, Wnt3a-CM was centrifuged at 10,000 × g for 10 min and filtered through a nitrocellulose membrane. The activity of Wnt3a-CM was assayed on L cells by examining the increase in β-catenin as described by Willert et al. (14). Wnt3a-CM was added to cells at a 20% final concentration in all experiments.

**Plasmids and Constructs—Myc-tagged LRPS was previously described (15). LRPS- and LRPS-derived truncated constructs were amplified by PCR and fused to the indicated tag-epitope into pcDNA3.1 vector. β-Catenin, disheveled, and Wnt3a cDNAs were obtained by reverse transcription-PCR and confirmed by nucleotide sequencing. Isolated sequences were tagged with the appropriate epitope and subcloned into the expression plasmid pcDNA3.1. The dominant negative form of disheveled (Ddd) was generated as described previously (16, 17). Stable mutant β-catenin (β-catenin*) was generated as described by Morin et al. (18). Axin expression vector was kindly provided Dr. D. L. Shi (CNRS, France). The Flag-Frat1 expression construct was kindly provided by Dr. E. Fraser (Institute of Cancer Research, United Kingdom). The Flag-negative form of Frat1, Frat1N, was generated as described by Li et al. (19). The expression of all tagged expression constructs was confirmed by Western blotting.

**Cell Cultures and Transfection—** COS-7 monkey kidney cells (ATCC) and mouse fibroblast L cells (ATCC) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Mesenchymal mouse C3H10T1/2 cells were grown in α-modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were plated in 24-well plates at 4 × 10^4 cells/well 24 h prior to transfection, then transiently transfected with the indicated construct (1 μg total DNA) using FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol. 18 h after transfection, cells were washed and cultured in medium at 2% fetal calf serum for an additional 48 h under the indicated conditions. Cells were then collected for luciferase assays, immunoprecipitation, or Western blotting.

When luciferase reporters were used, 20 ng of pRL-TK (Promega) encoding a Renilla luciferase gene downstream of a minimal herpes simplex virus-thymidine kinase promoter was systematically added to the transfection mix to assess transfection efficiency. Luciferase was determined by using Dual Luciferase Activity Kit (Promega) according to the manufacturer's instructions.

**Western Blot and Immunoprecipitation Assay—** For Western blot analysis and immunoprecipitation (IP), transiently transfected cells were harvested and lysed with M-PER Mammalian Protein Extraction Reagent (Pierce). Total protein content in cell lysates was estimated using the BCA protein assay (Pierce). To control protein expression, 10 μg of total protein were loaded on NuPAGE 4–12% Invitrogen). After electrophoresis, proteins were transferred onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences) and revealed with either horseradish peroxidase anti-FLAG (Sigma), horseradish peroxidase anti-epitope antibodies (Roche Diagnostics), horseradish peroxidase anti-GFP (Abcam), or anti-disheveled (rabbit polyclonal antibody, Chemicon). Anti-disheveled was detected using a secondary horseradish peroxidase anti-rabbit antibody (Sigma). Immunoreactive proteins were visualized by enhanced chemiluminescence detection according to the manufacturer (ECL + Amersham Biosciences). IP was carried out on 500 μg of total protein. Total proteins were incubated with 50 μl of μM protein G microbeads (Miltenyi Biotec) and 2 μg of indicated anti-epitope antibody at room temperature for 1 h. Immunoprecipitated proteins were eluted from the microcolumn with hot denaturant gel loading buffer and then subjected to Western Blot as described above.

**Confocal Microscopy Assay—** C3H10T1/2 cells were plated at 40,000 cells/well in a 6-wells plate with each well containing a sterile microcover glass, which can be removed for observation. 48 h after transfection or treatment, cells were fixed with 3.7% formaldehyde (Sigma) for 10 min, washed twice with phosphate-buffered saline, permeabilized with phosphate-buffered saline, 0.025% Triton X-100 (Sigma) for 5 min, and then incubated with phosphate-buffered saline, 3% bovine serum albumin for 15 min. After this, cells were incubated overnight at 4 °C with the primary antibody, a mouse monoclonal anti-FLAG (Santa Cruz), a rabbit polyclonal anti-β-catenin (Upstate Biotechnology), or a rabbit polyclonal anti-epitope antibodies (Santa Cruz) at the concentration of 5 μg/ml in phosphate-buffered saline. After being washed twice, cells were incubated with the secondary antibody, a goat anti-rabbit conjugated to fluorescein isothiocyanate (Santa Cruz, 2012), or a goat anti-mouse conjugated to rhodamine (Santa Cruz, sc2092) at 1/100 for 1 h at room temperature. The cover glasses were washed three times, mounted on glass slides, and viewed with a confocal laser-scanning microscope (LSM510, Carl Zeiss, Germany).

**Isolation of Fibroblastic Cells from Knock-out Lrp5 Mouse—** Murine fibroblastic calvaria cells were obtained from the calvariae of Lrp5<sup>−/−</sup> or their corresponding wild type littermate mice 1–2 days after birth by sequential collagenase digestion at 37 °C as described by Garcia et al. (20). Fibroblastic cells were transfected with indicated plasmids as described under “Cell Cultures and Transfection.”

**RESULTS**

**LRP5 Interacts with Frat1—** It has been clearly established that LRP5/6 receptor is required for Wnt to induce the canonical β-catenin signaling pathway (7–9). Interestingly, in the absence of Wnt, overexpression of LRPS lacking either the extracellular or both the transmembrane and extracellular domains leads to the activation of the canonical signaling pathway (data not shown (5)). These data strongly suggest that the cytoplasmic tail of LRPS is able to interact with intracellular players resulting in the activation of Wnt pathway. We have therefore performed a Y2H screen to identify proteins that interact with the LRPS cytoplasmic domain using two mouse embryo cDNA libraries (11 and 19 days). From each library, ~20 million clones were screened; clones showing interaction with LRPS bait in the primary screen were confirmed in secondary screen (see “Experimental Procedures”). Prey from confirmed clones were selected for the specificity of interaction with LRPS cytoplasmic tail by testing for interaction with two other unrelated baits (i.e. Id3 and Sprouty2) and for the absence of autoactivation. In summary, 35 and 77 clones were confirmed, respectively, from the 11-day and the 19-day embryonic cDNA library. Prey from confirmed clones were sequenced to identify the corresponding cDNA. Sequence analysis led to the identification of 45 distinct cDNAs, from which 85% were found in both libraries. Among them we identified frequently rearranged in advanced T-cell lymphoma 1 (Frat1) as a new potential partner for LRPS and further investigated the physiological significance of LRPS-Frat1 interaction. It is worth noting that Axin, which has been shown to interact with LRPS (Ref. 5 and our data in Fig. 7), was not pulled out from our two-hybrid screen.

**LRPS cytoplasmic domain (LRPS<sub>tail</sub>) and murine Frat1 were cloned in expression vectors as Myc or FLAG-tagged, respectively, and expression in COS-7 cells was verified by Western blotting (Fig. 1A). To confirm the interaction identified in Y2H, Myc-LRP5<sub>tail</sub> and Flag-Frat were co-expressed in COS-7 cells and found to co-immunoprecipitate (Fig. 1A). In addition, Flag-Frat1 was shown to co-immunoprecipitate with the Myc-LRP5<sub>full-length</sub> protein (see Fig. 5C). We also tested whether Frat1 interacts with LRPS, a close and functionally related homologue of LRPS. As shown in Fig. 1B, LRPS<sub>tail</sub> constructs also immunoprecipitated with Frat1 when both were co-expressed in COS-7 cells indicating that Frat1 interaction is not restricted to LRPS but also occurs with LRPS. Co-expression of Flag-Frat1 with two other unrelated Myc-tagged proteins (i.e. Id3, Sprouty2) did not allow co-immunoprecipitation (data not show). To further investigate the LRPS cytoplasmic tail domain involved in the interaction with Frat1 and to test whether the PPT/SP motif that was implicated in Axin interaction (5) is also involved in Frat1 interaction, we generated truncated LRPS<sub>tail</sub> constructs deleted of 28, 47, or 78 C-terminal residues named, respectively, LRPS<sub>tailΔ28</sub>, LRPS<sub>tailΔ47</sub>, and LRPS<sub>tailΔ78</sub>. These constructs were assessed for interaction with Frat1 by co-IP assay, and data clearly show that Frat1 interacts with LRPS<sub>tailΔ28</sub> (Fig. 1A) but not with LRPS<sub>tailΔ47</sub> (data not shown) and LRPS<sub>tailΔ78</sub> (Fig. 1A). These data strongly suggest that the interaction domain with Frat1 is
located in the last 47 amino acid residues of the LRP5 between residues 1569 and 1588 of the cytoplasmic tail. This interaction domain contains one PPSP motif, so this motif was mutated to two residues 1569 and 1588 of the cytoplasmic tail. This interaction was determined using yeast-two-hybrid (Y2H) assays. To evaluate the potential role of LRP5 and Frat1 interaction, we co-transfected cells with LRP5 lacking the extracellular domain (LRP5C) and Frat1. We used LRP5C instead of wild type LRP5, because overexpression of LRP5, unlike LRP5, does not induce significant Wnt signaling (Refs. 6 and 21 and data not shown). As shown in Fig. 2A, LRP5C acts as a constitutively active receptor and triggers the β-catenin pathway in the absence of Wnt proteins. LRP6C acts with LRP5C to activate the Wnt signaling pathway in primary cells from Lrp5 mice (Fig. 3A). These data indicate that LRP5 and Frat1 contribute to the same signaling pathway.

To further support these findings, we prepared primary fibroblastic cells from wild type Lrp5 heterozygous Lrp5 mice. As shown in Fig. 3A, Wnt3a was able to activate the Wnt signaling pathway in primary cells from Lrp5+/− mice, whereas cells from Lrp5−/− mice displayed no response (Fig. 3A). As expected, overexpression of Frat1 in cells derived from wild type mice (Lrp5+/−) induced Wnt signaling (Fig. 2B, II), but it completely blocked Lrp5-induced β-catenin nuclear translocation (Fig. 2B, III). These data indicate that LRP5 and Frat1 contribute to the same signaling pathway.
FRAT1 INTERACTS WITH LRP5

**Fig. 2. Effect of LRP5C-truncated mutant forms on FRAT1 activity.** A, COS-7 cells were transiently co-transfected with TCF-1 expression construct, TOPflash, and pTK-Renilla. Where indicated, FRAT1-expressing vector was added with empty vector (Vector), LRP5C, LRP5Δ28, LRP5Δ47, or LRP5Δ78. Control experiments were carried out by performing transfection with each of the used constructs alone. 18 h after transfection, the medium was replaced by fresh culture medium, and 24 h later luciferase activity was determined in cell lysates and normalized to Renilla signal. All experiments were performed in triplicate and repeated three times. Data ± S.D. from one representative experiment are presented (*, p < 0.01). B, COS-7 cells were transfected with control vector (I), LRP5C (II), LRP5Δ47 (III), FRAT1 (IV), LRP5C and FRAT1 (V), or LRP5Δ78 and FRAT1 (VI). 18 h after transfection, the medium was replaced by fresh culture medium and cultured for additional 24 h. Cells were immunostained for β-catenin expression using a mouse anti-β-catenin antibody and revealed by a goat anti-mouse antibody conjugated to rhodamine (red fluorescence). Fluorescent cells were visualized under confocal microscopy. The experiment was repeated three times, and photos from one representative experiment are shown.

**Fig. 3. LRP5 expression is necessary for FRAT1 activity.** Primary fibroblastic cells from wild type mice (lrp5+/+) or homozygous Lrp5 knock-out mice (lrp5−/−) were transiently co-transfected with TCF-1 expression construct, TOPflash, and pTK-Renilla. Empty control vector, FRAT1 or constitutively active β-catenin (β-catenin+) expression constructs were added to the transfection mix when indicated. 18 h after transfection, the medium was replaced by fresh culture medium. Cells transfected with empty vector were left untreated (CTRL) or treated with Wnt3a-CM (Wnt3a), and NaCl at 20 mM or LiCl at 20 mM. 24 h later luciferase activity was determined in cell lysates and normalized to Renilla signal. All experiments were performed in triplicate and repeated three times. Data ± S.D. from one representative experiment are presented (p < 0.01).

FRAT1 has been shown to be localized into different cell compartments (22, 23). We tested the effect of LRP5C on FRAT1 cell distribution by immunohistochemistry using confocal microscopy. Overexpressed FRAT1 predominantly localized in the cytoplasm, yet a significant fraction (~25%) was also found on the cell membrane (Fig. 5, A I and B). The stimulation of cells with Wnt3a induced a shift in FRAT1 cell localization from the cytoplasm to the membrane, where over 80% of FRAT1 could be localized (Fig. 5, A II and B). A similar effect was obtained when LRP5C or LRP5CΔ28 were overexpressed in the presence of FRAT1 (Figs. 5A, III–IV and 7B). Overexpression of LRP5CΔ47 caused a reduction of FRAT1 levels on the membrane and induced a small but significant translocation of FRAT1 into the nucleus (~10%, Fig. 5B). Surprisingly, LRP5CΔ78, which we have shown to inhibit FRAT1 activity, induces the majority of FRAT1 to be localized into the nucleus (Fig. 5A V). The percentage of membrane, cytoplasm, or nucleus location of FRAT1 was determined, and the data are shown in Fig. 5B. In summary, data presented herein strongly suggest that the interaction of LRP5 with FRAT1 modulates Wnt/LRP5 signaling by regulating FRAT1 cellular localization.

DOMINANT NEGATIVE DVL DOES NOT INTERFERE WITH LRP5C/FRAT1 INTERACTION—FRAT1 has been shown to interact with several other components of the Wnt/β-catenin pathway, including DVL and GSK-3 (19, 24). We have investigated how DVL might be involved in LRP5/FRAT1 interaction and their ability to transduce a signal. The overexpression of DVL has not affected LRP5C or LRP5/FRAT1 interaction (data not shown). We then tested the effect of Xdd, a DVL mutant with an internal deletion in the PDZ domain that acts as a dominant negative (16, 17). The overexpression of Xdd strongly inhibits TCF-1 transcriptional activity induced by Wnt3a (Fig. 6A). The activity of FRAT1 was significantly but slightly inhibited by Xdd, suggesting that in overexpression conditions FRAT1 activity is largely independent of DVL. Interestingly, Xdd overexpression did not affect LRP5C or LRP5CΔ28 activity (Fig. 6A). Our results confirm previously reported data demonstrating that LRP5C acts in a DVL-independent manner (25). We next assessed whether Xdd could interfere in FRAT1 interaction with either LRP5 or LRP5C. As shown in Fig. 6B, although Xdd had no effect on LRP5C and FRAT1 interaction, it inhibits the interaction be-

activity (Fig. 3B). These data provide evidence of an intact Wnt signaling pathway in the Lrp5−/− cells and strongly suggest that LRP5 is required for FRAT1 to induce canonical Wnt signaling. We next addressed whether FRAT1 is involved in the constitutive activity of LRP5C. To answer this question we made a FRAT1 construct with the C-terminal region deleted (FRAT1N) that has been described to be capable of inhibiting Wnt activity (19). As expected, FRAT1N almost completely inhibited Wnt3a activity (Fig. 4A) and in addition it significantly inhibited LRP5C activity (Fig. 4B). Co-IP assays clearly indicate that FRAT1N is no longer able to interact with LRP5C (Fig. 4C) or LRP5 (data not shown). Furthermore, overexpression of FRAT1N blocked β-catenin nuclear translocation induced by LRP5C (data not shown). These data demonstrate that the FRAT1 interaction domain is located in the C-terminal part and suggest that FRAT1 is necessary for the constitutive activity of LRP5C.

LRP5 Signaling Controls FRAT1 Cellular Localization—FRAT1 has been shown to be localized into different cell compartments (22, 23). We tested the effect of LRP5C on FRAT1 cell distribution by immunohistochemistry using confocal microscopy. Overexpressed FRAT1 predominantly localized in the cytoplasm, yet a significant fraction (~25%) was also found on the cell membrane (Fig. 5, A I and B). The stimulation of cells with Wnt3a induced a shift in FRAT1 cell localization from the cytoplasm to the membrane, where over 80% of FRAT1 could be localized (Fig. 5, A II and B). A similar effect was obtained when LRP5C or LRP5CΔ28 were overexpressed in the presence of FRAT1 (Figs. 5A, III–IV and 7B). Overexpression of LRP5CΔ47 caused a reduction of FRAT1 levels on the membrane and induced a small but significant translocation of FRAT1 into the nucleus (~10%, Fig. 5B). Surprisingly, LRP5CΔ78, which we have shown to inhibit FRAT1 activity, induces the majority of FRAT1 to be localized into the nucleus (Fig. 5A V). The percentage of membrane, cytoplasm, or nucleus location of FRAT1 was determined, and the data are shown in Fig. 5B. In summary, data presented herein strongly suggest that the interaction of LRP5 with FRAT1 modulates Wnt/LRP5 signaling by regulating FRAT1 cellular localization.

Dominant Negative Dvl Does Not Interfere with LRP5C/FRAT1 Interaction—FRAT1 has been shown to interact with several other components of the Wnt/β-catenin pathway, including DVL and GSK-3 (19, 24). We have investigated how DVL might be involved in LRP5/FRAT1 interaction and their ability to transduce a signal. The overexpression of DVL has not affected LRP5C or LRP5/FRAT1 interaction (data not shown). We then tested the effect of Xdd, a DVL mutant with an internal deletion in the PDZ domain that acts as a dominant negative (16, 17). The overexpression of Xdd strongly inhibits TCF-1 transcriptional activity induced by Wnt3a (Fig. 6A). The activity of FRAT1 was significantly but slightly inhibited by Xdd, suggesting that in overexpression conditions FRAT1 activity is largely independent of DVL. Interestingly, Xdd overexpression did not affect LRP5C or LRP5CΔ28 activity (Fig. 6A). Our results confirm previously reported data demonstrating that LRP5C acts in a DVL-independent manner (25). We next assessed whether Xdd could interfere in FRAT1 interaction with either LRP5 or LRP5C. As shown in Fig. 6B, although Xdd had no effect on LRP5C and FRAT1 interaction, it inhibits the interaction be-
tween full-length LRP5 and Frat1 as determined by co-IP as-
say. Furthermore, Xdd overexpression did not inhibit LRP5C-
induced Frat1 membrane recruitment, whereas it totally
blocked full-length LRP5-mediated Frat1 membrane localiza-
tion (data not shown). Collectively, those data suggest that
although Dvl affects LRP5/Frat1 interaction and subsequent
Wnt signaling, it is unable to behave similarly with LRP5
deprieved of its extracellular domain, thus explaining the fact
that LRP5C is acting independently of this major Wnt signal-
ing player.

**DISCUSSION**

Although it has been clearly established that LRP5/6 is a
major actor in the activation of the Wnt canonical pathway (for
review see Ref. 28), the precise molecular mechanisms by
which LRP5/6 participate in this important cascade remain to
be elucidated. Currently there is no clear evidence of a binding
of any Wnt protein to LRP5/6, and there are only a few dem-
strations on how LRP5/6 transduces the intracellular signal
leading to the activation of Ncadherin pathway. In fact, unlike
other low density lipoprotein-related receptors (e.g. low density
lipoprotein receptor), the cytoplasmic tail of LRP5 displayed no
NPXY motif that was identified to interact with the adaptor
protein Dab-1 (29). The fact that the LRP5/6 cytoplasmic tail is
rich in proline residues suggests that it might be able to bind a
SH3-like motif, but nobody has investigated such a possibility.
Today the only protein shown to be able to interact with the
LRP5 cytoplasmic domain is Axin (5, 26). By performing a Y2H
screen to identify additional partners for the LRP5 receptor, we
have identified Frat1, and we herein present evidence of the
crucial role that this interaction plays in the activation of the
canonical Wnt/β-catenin cascade.
Frat1 (also named GBP for GSK-3-binding protein) was first identified in Xenopus as a protein that inhibits GSK-3 in vivo, appearing to act as a positive regulator of the Wnt signaling pathway by stabilizing β-catenin (30). Further studies performed in Xenopus showed that Frat1 inhibited GSK-3 activity toward β-catenin, at least in part, by preventing Axin binding to GSK-3 (24, 31, 32). Transfection studies in mammalian cells have confirmed the role of Frat1 in the stabilization of β-catenin and have shown the presence of Frat1 in complexes with Dvl, GSK-3, and Axin (19). Taken together, these findings suggest that Wnt signaling causes a recruitment of Frat1 into such complexes, leading to the Frat1-mediated dissociation of GSK-3β from Axin. To study the function of Frat1 in vivo, Jonkers et al. (33) generated a Frat1-deficient mouse, which displays an apparently normal, healthy, and fertile phenotype. However, one cannot draw conclusions from this model as to the dispensability of Frat1 for Wnt signaling, because other Frat proteins (i.e. Frat2 and Frat3) that are functionally and structurally similar (34, 35) can compensate the loss of Frat1 expression. Indeed, Jonkers et al. (33) show that the compensatory activity of Frat3 accounts for the lack of phenotype in Frat1-deficient mice.

In the present study we clearly demonstrate that Wnt3a induces the localization of Frat1 at the membrane through the binding to LRP5 cytoplasmic tail. The interaction of Frat1 with LRP5 is required to activate the canonical β-catenin signaling pathway. Mao et al. (5) have shown that LRP5 interacts with Axin and recruits it to the membrane. Very recently, Tamai et al. (27) have presented evidence that the PPS/TP motifs within the LRP5/6 cytoplasmic tail are the docking sites for Axin and that only one PPS/TP motif is sufficient to drive the interaction with Axin. Results presented herein show that the region required for Frat1 binding and recruiting to the membrane is located in the 47 C-terminal amino acids of LRP5. Although the LRP5 region involved in Frat1 binding contains three repeated PPT/SP motifs, our data show that, unlike for Axin, this motif is not involved in LRP5 binding to Frat1. In fact, a mutation of the PPSP motif does not affect LRP5/Frat1 interaction. Surprisingly, overexpression of LRP5CΔ78 that does not display interaction with Frat1 resulted in the accumulation of Frat1 in the cytoplasm, therefore depriving Frat1 of one of its usual partner proteins. Under these conditions Frat1 can translocate into the nucleus and inhibit its activity.

**FIG. 6.** Effect of disheveled dominant negative mutant on Frat1/LRP5C activity and association. A, C3H10T1/2 cells were transfected with TCF-1 expression construct, TOPflash, and pTK-Renilla. Where indicated LRP5C, LRP5CΔ28, or LRP5CΔ78 was added in the presence or absence of disheveled dominant negative (Xdd). When indicated, cells were treated with Wnt3a-CM (Wnt3a). 18 h after transfection the medium was replaced by fresh culture medium, and 24 h later luciferase activity was determined in cell lysates and normalized to Renilla signal. All experiments were performed in triplicate and repeated three times. Data ± S.D. from one representative experiment are presented (*, p<0.01). Cells were co-transfected with Frat1-Flag and LRP5C-Myc (B) or LRP5-Myc (C) in absence or presence of the Xdd expression construct. Immunoprecipitation (IP) on total cell lysates using anti-Myc antibody was performed. Total cell lysates and immunoprecipitates were analyzed by Western blotting using indicated antibodies. Arrows indicate the expected band size.

**FIG. 7.** Axin interacts in the same complex with Frat1 and LRP5 interaction. COS-7 cells were transiently transfected with Axin-Myc expression vector with Frat1-Flag, LRP5C-GFP, or both expression constructs. Cells lysates were immunoprecipitated (IP) with anti-FLAG or anti-GFP antibodies. Immunoprecipitates were analyzed by Western blotting using anti-GFP, anti-Myc, or anti-FLAG antibodies. Total cell lysates were analyzed by Western blotting using anti-GFP, anti-Myc, or anti-FLAG antibodies (lower box). Arrows indicate the size of expected band.

Frat1 Interacts with LRP5
Our co-IP experiments show that a dominant negative form of Dvl, which inhibits Wnt-induced TCF-1 transcriptional activation and subsequent inhibition of GSK-3β activity (22). Phosphorylation of the PPT/SP motifs confers Axin binding, but it remains to be determined whether this is also required for Frat1 binding (27). Our results demonstrate that both Frat1 and Axin interact with the LRP5 cytoplasmic tail, but they most likely do so on distinct motifs. These findings strongly suggest that following Wnt stimulation, Frat1 is recruited to the membrane where it interacts with LRP5 in the same complex as Axin thus enabling it to mediate dissociation of GSK-3 from Axin with the subsequent stabilization of β-catenin (Fig. 8). It remains to be determined whether GSK-3 is indeed involved in this complex. Additional investigations are therefore required to clarify these possibilities.

Our data show that LRP5/Frat1 interaction is required for the β-catenin signaling. This was clearly demonstrated using fibroblastic cells derived from LRP5-knock-out animals. Furthermore, LRP5 with the C-terminal polypeptide truncated in the Frat1 interaction inhibits Frat1-mediated transcriptional activity and more interestingly induces the nuclear localization of Frat1. In this work, in the absence of Wnt or LRP5C overexpression, Frat1 was able to induce TCF-1 transcriptional activation and was located predominantly in the cytoplasm. From our data one could conclude that nuclear localization of Frat1 was concomitant with its loss of activity toward TCF-1. Franca-Koh et al. (22) have determined a nuclear export sequence within the Frat1 amino acid sequence and showed that Frat1 regulates GSK-3β nuclear export; however, the precise role of Frat1-mediated GSK-3 nuclear export in Wnt signaling has not been investigated.

The precise mechanism that underlies the constitutive activity LRP5C remains currently unclear. Our data and those from Mao et al. (5) strongly suggest that LRP5C activates the β-catenin pathway in a Dvl-independent manner. Dvl, an important regulator of the Wnt canonical pathway, is known to interact with both Frat1 and Axin to regulate β-catenin stabilization (19). Our co-IP experiments show that a dominant negative form of Dvl, which inhibits Wnt-induced TCF-1 transcriptional activation, is unable to disrupt the LRP5/Frat1 interaction. On the opposite side, the dominant negative form of Dvl inhibits the LRP5/Frat1 interaction. These data demonstrate that Dvl is not involved in LRP5C constitutive activity, because it is unable to modulate Frat1 interaction with this truncated form of LRP5. Liu et al. (21) have demonstrated that LRP5/6 forms an inactive homomeric complex, whereas constitutively active LRP5/6 mutants (LRP5/6C) were monomeric, and their activity is inhibited by forced dimerization. These findings suggest that Wnt induces a conformational switch in the LRP5/6 extracellular domain that relieves the allosteric inhibition imposed on the intracellular domain. In addition to the model proposed by Liu et al. (21), very recent data published by Cong et al. (36) has clearly demonstrated that Wnt protein binds to both LRP and Frizzled extracellular domains, forming membrane-associated hetero-oligomers. This membrane-associated receptor complex interacts with both disheveled, via the intracellular portions of Frizzled, and Axin and Frat1 (based on herein presented data) via the intracellular domain of LRP. However, functional and biochemical studies presented herein and by Mao et al. (5) show that the constitutive activity of the LRP5 mutant, LRP5C, is not fully comparable with the Wnt activation through LRP/Frizzled membrane-associated receptor. Altogether, these data suggest that the conformational alteration of LRP5 intracellularly induced by truncating the extracellular domain is slightly distinct from that induced by Wnts.

In summary, based on the data presented in this report and observations reported previously, we propose a conceptual model depicted in Fig. 8 for Wnt signaling through the LRP5 receptor. Following Wnt activation, a conformational modification of the LRP5 cytoplasmic domain occurs, allowing the recruitment of Frat1 to the membrane. The Frat1 membrane localization put it into proximity of Dvl, which interacts with Frizzled receptors. The activated status of the LRP5 receptor is also able to translocate Axin to the membrane where it interacts with Frat1. Axin recruitment to the membrane prevents it from enhancing the degradation of β-catenin. In the absence of Wnts, Axin is found within a protein complex that includes APC, GSK-3β, and β-catenin. One could hypothesize that the LRP5/Axin/Frat1 interaction in the presence of Wnts will bring GSK-3β close to Frat1 and thus allow Frat1/GSK-3β interaction and subsequent inhibition of GSK-3β by Frat1 leading to the stabilization of β-catenin.

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