The Mechanism of Endogenous Receptor Activation Functionally Distinguishes Prototype Canonical and Noncanonical Wnts

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Wnt glycoproteins are developmentally essential signaling molecules, and lesions afflicting Wnt pathways play important roles in human diseases. Some Wnts signal to the canonical pathway by stabilizing β -catenin, while others lack this activity. Frizzled serpentine receptors mediate distinct signaling pathways by both classes of Wnts. Here, we tandemly linked noncanonical Wnt5a with the C-terminal half of Dickkopf-2 (Dkk2C), a distinct ligand of the Wnt coreceptor LRP5/6. Whereas Wnt5a, Dkk2C, or both together were incapable of stimulating endogenous canonical signaling, the Wnt5a/Dkk2C chimera efficiently activated this pathway in a manner inhibitable by specific antagonists of either frizzled or LRP receptors. Thus, activation of the canonical pathway requires ligand coupling of an endogenous frizzled/LRP coreceptor complex, rather than Wnt triggering each receptor independently. Moreover, fusion of Wnt5a with Dkk2C unmasked its ability to signal to Dishevelled through multiple frizzleds, indicating that the lack of functional interaction with LRP distinguishes noncanonical Wnt5a from canonical Wnts in mammalian cells. These findings provide a novel mechanism by which the same receptor can be switched between distinct signaling pathways depending on the differential recruitment of a coreceptor by members of the same ligand family.

The Wnt family of secreted glycoproteins is a major group of developmentally important signaling molecules which play essential roles in embryonic induction, cell polarity generation, and cell fate specification in metazoan species from hydra to humans (33, 35, 57). Up to 19 members of the Wnt family have been identified in mammals to date and classified into two classes based on their signaling functions (11, 24, 43). The canonical Wnt class contains many Wnt proteins, including Wnt1, Wnt2, Wnt3, Wnt3a, and Wnt-8, which activate the well-conserved canonical Wnt signaling pathway, leading to stabilization of β -catenin (33). Accumulation of β -catenin results in its nuclear translocation and activation of lymphoid enhancer factor/T-cell factor (Lef/Tcf) transcription factors (12). These Wnts are also capable of inducing secondary axes when ectopically expressed in *Xenopus laevis* embryos. In addition to essential roles in embryonic development, canonical Wnt signaling is also involved in the normal homeostasis of stem cells of multiple tissue origins including colon epithelial stem cells (51) and hematopoietic stem cells (40, 56). Finally, aberrant activation of the canonical Wnt pathway has been found to frequently occur in human cancers (38).

Certain so-called noncanonical Wnts, such as Wnt5a and Wnt11, fail to signal through β -catenin or induce axis duplication in *Xenopus* embryos (11, 43) but instead interfere with the morphogenetic movements of the convergent extension process that occurs during gastrulation (11, 17, 45). Noncanonical Wnts also play essential organizing roles in development. Targeted gene disruption in the mouse has shown that Wnt5a is required for the elongation of axial structures and may influence the proliferation of precursor cells (59). Wnt5a is fre-

quently overexpressed in several cancers (19, 25) and promotes cell motility and invasion of metastatic melanoma cells (53). Despite its proposed oncogenic function in certain tissues, Wnt5a inhibits B-cell proliferation and has been suggested to function as a tumor suppressor in hematopoietic malignancies (27). Thus, the roles of Wnt5a in tumorigenesis may be cellular context dependent.

Both classes of Wnt ligands transduce signals through membrane receptors, which have been identified to be the multimer frizzled family of serpentine receptors. All 10 known mammalian frizzleds share an extracellular conserved cysteine-rich domain for Wnt ligand binding (5, 52, 57). However, the affinities between frizzled family members and different Wnt ligands vary (5, 41), suggesting specific functional Wnt-frizzled pairings. Frizzled receptors have been shown to transmit at least three distinct signaling pathways. In addition to the canonical β -catenin pathway (5) and the Rho/JNK planar cell polarity pathway (34), binding of Wnt5a to several frizzleds has been reported to signal through heterotrimeric G proteins and trigger the release of intracellular Ca^{2+} (44), which activates protein kinase C and calmodulin-dependent protein kinase II (23) and inhibits the β -catenin signaling cascade (20, 23, 49).

Recent studies have uncovered that the single transmembrane coreceptors LRP5 and LRP6, or the *Drosophila melanogaster* ortholog arrow, are required for canonical Wnt signaling (37, 46, 54). LRP5/6 also serve as receptors for the Dickkopf (Dkk) family of Wnt antagonists (4, 31, 42). Dkk-1, Dkk-2, and Dkk-4 have been shown to inhibit canonical Wnt signaling through binding to LRP5/6 and another receptor, Kremen (30), and these antagonists do not interact with frizzled receptors (4, 31, 42). The LRP5/6 interaction domain of Dkk has been mapped to the C-terminal domain. Of note, the C-terminal domain of Dkk-1 and Dkk-2 has been reported to activate canonical β -catenin signaling through exogenously expressed LRP6 receptors (7, 26). Unlike Dkk, Wnt ligands such

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as Wnt1 bind to the extracellular domains of both frizzled and LRP6 receptors, independently, by which a coreceptor complex can form in vitro (46). However, how these two types of receptors coordinate in vivo to activate canonical Wnt signaling is not yet known.

In the present study, we employed a chimeric ligand strategy to tandemly link noncanonical Wnt5a with the C-terminal domain of Dickkopf-2 (Dkk2C). Whereas Wnt5a, Dkk2C, or both together were incapable of stimulating endogenous canonical signaling, the Wnt5a/Dkk2C chimera efficiently activated this pathway in a manner similar to that induced by the canonical Wnt3a. Our results establish that the activation of the canonical β -catenin pathway requires ligand coupling of an endogenous frizzled/LRP coreceptor complex, rather than Wnt triggering each receptor independently. Moreover, fusion of Wnt5a with Dkk2C unmasked its ability to signal to Dishevelled through multiple frizzleds, indicating that the lack of functional interaction with LRP distinguishes noncanonical Wnts from canonical Wnts.

MATERIALS AND METHODS

Constructs. Flag-tagged mouse Wnt3a and Wnt5a constructs were generated by PCR amplification of full-length cDNAs which were inserted into a pFlag-CMV1 vector containing an N-terminal signal peptide followed by the Flag epitope sequence. To generate the Dkk2C constructs, primers flanking the C-terminal domain of mouse Dkk-2 from residues 141 to 259 were used to amplify a PCR fragment from a pool of mouse embryonic cDNAs. The amplified cDNA fragment was then inserted into the pFlag-CMV1 vector downstream of the signal peptide and Flag epitope to generate the Dkk2C construct or downstream of Wnt5a (deleted stop codon) to generate the chimeric Wnt5a/Dkk2C construct. The Wnt5a/green fluorescent protein construct was generated as a negative control. Flag-tagged LRP6 Δ C was generated by PCR to delete the intracellular domain of LRP6 and inserted into pFlag-CMV1. Flag-tagged FRP1, LRP6, and Δ N-LRP6 constructs have been previously described (2, 28). The rat frizzled-1 (rfz1); mouse fz2, fz3, fz4, fz6, fz7, fz8; and human fz5 (Hfz5) mammalian expression constructs were kindly provided by J. Kitajewski (Columbia University). The mouse fz9 expression construct was kindly provided by U. Francke (Stanford University). Mouse fz10 cDNA containing the entire coding region was amplified by PCR from a mouse embryonic cDNA pool and cloned into the pFlag-CMV1 expression vector.

RNA interference. The 19-nucleotide short hairpin oligonucleotides were expressed from an H1 promoter in the pSUPER vector (kindly provided by R. Agami) to obtain RNA interference (8) against the three Dvl homologues and human LRP6 receptor. The short hairpin RNA interfering oligonucleotide sequences CAAGATCACCTTCTCCGAG and CTTTGAGAACATGAGCAAC utilized to target human Dvl-1 and Dvl-3 or human Dvl-2, respectively, were previously reported (26). The short hairpin oligonucleotide sequence CCGCATGGTGATTGATGAA was utilized to target the human LRP6 receptor (3).

Cell culture and transfection. 293T human embryonic kidney cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transient transfection was performed by using Eugene (Roche) according to the manufacturer's instructions.

Uncomplexed β -catenin analysis, immunoprecipitation, and immunoblotting. The glutathione *S*-transferase (GST)-E-cadherin binding assay was performed as described previously (2). Briefly, bacterially expressed GST-E-cadherin was purified with glutathione-Sepharose beads and incubated with 1 mg of each cell lysate. GST-E-cadherin/ β -catenin complexes bound to the beads were recovered by centrifugation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with α - β -catenin antibody (Transduction Laboratories). Cell lysate preparation, immunoprecipitation, and immunoblotting were performed as described previously (28).

Luciferase reporter assays. Luciferase assays in 293T cells were done in 6-well plates in triplicate as previously described (28). The following plasmid concentrations were used (nanograms per well): pTOPFLASH, 500; pCMV-Renilla, 5; mouse Wnt3a, 100; Wnt5a, 100; Dkk2C, 100; frizzleds, 25; and LRP6, 200, unless otherwise indicated. Luciferase activity was normalized against Renilla activity. To analyze Wnt signaling in a paracrine mode (13), 293T target cells in 6-well plates were transfected with Topglow TCF luciferase reporter (500 ng) and

pRL-CMV (5 ng). Concomitantly, the effector 293T cells were seeded at 3×10^6 cells in 9-cm-diameter petri dishes and transfected with either empty vector, Wnt5a, Dkk2C, Wnt5a plus Dkk2C, or Wnt5a/Dkk2C (2 μ g of each construct). Six hours following transfection, the effector transfectants were washed twice with phosphate-buffered saline (PBS), trypsinized, and seeded at a ratio of 2- to 3-fold onto the PBS-prewashed target transfectants. After 48 h of cocultivation, cells were lysed and assayed for luciferase activity.

Membrane biotinylation assay. Cell surface protein biotinylation was performed as described previously (10). Briefly, cells were washed twice with cold PBS (pH 8.0) and suspended in 0.5 mg of Sulfo-NHS-LC-Biotin (Pierce)/ml at room temperature for 30 min. After being washed three times with 100 mM glycine-PBS, cells were lysed with radioimmunoprecipitation assay buffer, and cell lysates were incubated with immobilized Avidin beads (Pierce) for 3 h. Beads were washed three times, and the bound proteins were eluted with Laemmli buffer for immunoblot analysis with anti-Flag antibody.

Reverse transcription (RT)-PCR. Total RNA was isolated with Trizol reagent (Invitrogen) and treated with RNase-free DNase. A total of 2.5 μ g of total RNA was reverse transcribed from oligo(dT) primer, and the cDNA was amplified with primers specific for human fz2, fz4, fz5, LRP5, and LRP6 under the following conditions: 35 cycles of 95°C for 30s, 48°C for 1 min, and 72°C 1 min, followed by a 7-min extension at 72°C. The primer sequences were as follows: Hfz2 forward, 5'-CACCATCATGAAGCAGAC-3'; Hfz2 reverse, 5'-CTCACACG GTGGTCTCAC-3'; Hfz4 forward, 5'-ACCAAGGCAGCATCTAGCAG-3'; Hfz4 reverse, 5'-ACTACAGTCGGCACTCAATA-3'; Hfz5 forward, CTACCA CAAGCAGGTGTC-3'; Hfz5 reverse, 5'-AATTGAGACCACACAGTTCA-3'; LRP5 forward, 5'-GCTGTGAATGTGGCCAAGGTCGTC-3'; LRP5 reverse, 5'-CACCAGGATGCCATCAGCAAGTC-3'; LRP6 forward, 5'-AGGCTACA AATGTTTCATCGAGTGA-3'; LRP6 reverse, 5'-GCAGGAAAGTCGTAGGA GCACTAC-3'.

RESULTS

Lack of canonical β -catenin signaling ability of Wnt5a through multiple frizzled receptors. To investigate the underlying mechanism distinguishing signaling by prototype canonical and noncanonical Wnts in mammalian cells, we chose mouse Wnt3a and Wnt5a as representatives of these two respective classes and compared their β -catenin signaling abilities in 293T cells. As shown in Fig. 1a, Wnt3a expression induced a dramatic increase in uncomplexed, stabilized β -catenin, whereas Wnt5a had no detectable effect, consistent with previous studies (43). Several frizzled receptors, including fz2 (44), fz4 (9), and fz5 (15), were reported to transduce Wnt5a signal to the Ca^{2+} or β -catenin pathway. To determine whether lack of canonical signaling by Wnt5a in 293T cells was due to the absence of these frizzleds, we performed RT-PCR analysis. Figure 1b shows that 293T cells expressed fz2 and fz4 transcripts, but fz5 was undetectable. Hfz5 has been reported to transduce the *Xenopus* Wnt5a (XWnt5a) signal to the canonical pathway in *Xenopus* embryos (15). However, when we ectopically expressed Hfz5 with Wnt5a in 293T cells, this receptor failed to couple Wnt5a to the β -catenin pathway (Fig. 1c). The TOPFLASH TCF reporter assay was next performed for Wnt5a in combination with all 10 exogenously expressed frizzleds (Fig. 1d), but none was able to transduce a β -catenin signaling response. In contrast, Wnt3a stimulated TCF signaling in cooperation with several frizzleds including rfz1, fz2, Hfz5, fz7, and fz8. These findings indicated that the inability of Wnt5a to signal to the canonical β -catenin pathway was not due to the absence of frizzled receptors in 293T cells.

Differential signaling responses of LRP6 receptors to Wnt3a, Wnt5a, and Dkk2C. The identification of LRP5/6 proteins as Wnt coreceptors in β -catenin signaling prompted us to assess whether LRP receptor overexpression might be sufficient to couple Wnt5a to canonical signaling, although endog-

FIG. 1. Lack of ability of Wnt5a to activate canonical Wnt signaling. (a) Comparison of Wnt3a and Wnt5a effects on β -catenin stabilization. One microgram of vector, Wnt3a, or Wnt5a construct was transfected into 293T cells, and the uncomplexed β -catenin (β -cat) assay was performed after 48 h. Wnt expressions were detected by anti-Flag (α -Flag) antibody. (b) RT-PCR analysis of Wnt receptors in 293T cells. –RT, amplification in the absence of reverse transcriptase as a negative control. (c) Wnt5a does not activate the canonical β -catenin pathway through Hfz5. One microgram of each construct was transfected into 293T cells, and uncomplexed β -catenin levels were measured 48 h later. Hfz5 functional expression was confirmed by its activity in the TCF reporter assay (see below). (d) Comparison of Wnt3a and Wnt5a in TOPFLASH TCF signaling through different frizzleds. The values represent the means ($\pm$ standard deviations) of two independent experiments performed in triplicate and are expressed in relative luciferase units (RLU).

enous LRP5 and LRP6 were both detectable at the transcript level by RT-PCR in 293T cells (Fig. 1b). Figure 2a shows that while Wnt3a alone stabilized β -catenin and cooperated with ectopic LRP6 to induce higher β -catenin level, Wnt5a showed no effects on β -catenin stabilization when expressed even with overexpressed LRP6, suggesting that Wnt5a may not be a functional ligand for LRP6. The C-terminal domain of Dkk-2 (Dkk2C) binds to LRP5/6 (7) and activates canonical signaling through exogenously expressed LRP6 (7, 26). Consistent with these observations, we found that when expressed with ectopic LRP6 in 293T cells, Dkk2C induced detectable β -catenin stabilization (Fig. 2a) and TCF reporter activity (Fig. 2b), although to a much lesser extent than that observed with Wnt3a. However, Dkk2C alone induced no detectable β -catenin signaling (Fig. 2a and b), indicating that endogenous LRP5/6 receptor levels were insufficient to activate canonical signaling in response to Dkk2C.

We previously demonstrated that exogenously expressed LRP6 receptors form inactive oligomers through their extracellular domains and that Wnts alter the intracellular conformation to allow Axin recruitment (28). This conformational change could be demonstrated by the ability of Wnts to inhibit the constitutive tyrosine autophosphorylation of an LRP6-fibroblast growth factor receptor (FGFR) chimera containing the FGFR tyrosine kinase domain (28). We employed this chimera to test the functional interaction of Wnt5a with LRP6. Figure 2c shows that Wnt3a dramatically inhibited LRP6-

FIG. 2. Differential canonical signaling responses of LRP6 to Wnt3a, Wnt5a, or Dkk2C. (a) Comparison of β -catenin (β -cat) stabilization induced by Wnt3a, Wnt5a, and Dkk2C in response to exogenous LRP6. One microgram of each construct was transfected into 293T cells. Uncomplexed β -catenin was measured after 48 h. Vec, vector; α -Flag, anti-Flag antibody. (b) Differential TCF reporter activation by Wnt3a, Wnt5a, and Dkk2C in response to exogenous LRP6. RLU, relative luciferase units. (c) Effects of Wnt3a, Wnt5a, and Dkk2C on constitutive tyrosine phosphorylation of the LRP6-FGFR chimera. One microgram of Flag-tagged LRP6-FGFR was cotransfected with 4 μ g of vector, Wnt3a, Wnt5a, or Dkk2C into 293T cells. At 48 h, 1 mg of each cell lysate was immunoprecipitated (IP) with anti-Flag M2 beads followed by immunoblotting with anti-p-Tyr. The same blot was stripped and reprobed with anti-Flag for detection of LRP6-FGFR protein levels. The results shown are representative of three independent experiments. The relative intensity of each phosphorylated LRP6-FGFR band was normalized against the corresponding LRP6-FGFR protein band following densitometry analysis. Expression of transfected proteins was detected with anti-Flag antibody in a and c.

FGFR tyrosine autophosphorylation as previously demonstrated (28). Dkk2C similarly reduced tyrosine phosphorylation, although to a lesser extent, consistent with its relative ability to stimulate canonical signaling through exogenous LRP6 (Fig. 2a). In striking contrast, Wnt5a caused no obvious alteration in LRP6-FGFR tyrosine autophosphorylation. Thus, the lack of functional interaction between Wnt5a and LRP6 was a likely mechanism for its inability to act as a canonical Wnt ligand.

Wnt5a/Dkk2C chimeric ligand activates canonical Wnt signaling through multiple frizzled/LRP coreceptors. The involvement of both frizzled and LRP receptors in canonical Wnt signaling, along with the fact that Wnt5a was unable to activate LRP6 receptors (Fig. 2), seemed likely to explain why Wnt5a belongs to the noncanonical Wnt ligand class. However, it was still possible that Wnt5a might also lack the ability to interact with frizzleds in a manner required for canonical sig-

FIG. 3. A Wnt5a/Dkk2C chimeric ligand efficiently activates canonical β -catenin signaling. (a) Induction of β -catenin stabilization by expression of Wnt5a/Dkk2C alone (left panel) or with ectopic LRP6 (right panel). One microgram of each construct was transfected into 293T cells followed by uncomplexed β -catenin analysis after 48 h. Vec, vector; α - β -cat, anti- β -catenin antibody; α -Flag, anti-Flag antibody. (b) Detection of secretion of Wnt5a and Wnt5a/Dkk2C in 293T cells through surface biotinylation assay. One microgram of Flag-tagged Wnt5a or Wnt5a/Dkk2C construct was transfected into 293T cells. Cell surface biotinylation assay was performed 48 h later. (c) Specific antagonists of frizzled and LRP receptors inhibit canonical signaling by Wnt5a/Dkk2C. Upper panel, FRP and LRP6 Δ C inhibit Wnt5a/Dkk2C-induced β -catenin stabilization. One microgram of Wnt5a/Dkk2C was cotransfected with 1 μ g of vector, FRP, or LRP6 Δ C in 293T cells. Lower panel, short hairpin RNA against LRP6 (shLRP6) inhibits β -catenin stabilization induced by Wnt5a/Dkk2C. One microgram of Wnt5a/Dkk2C was cotransfected with 2.5 μ g of control vector or pSuper-shLRP6. Uncomplexed β -catenin was measured after 48 h. The specificity and effectiveness of the shLRP6 have been proven previously by targeting the exogenously expressed Flag-LRP6 receptors (3). (d) Dvl is required for canonical signaling induced by Wnt5a/Dkk2C. A total of 1.5 μ g of Wnt3a, Wnt5a/Dkk2C, or Δ N-LRP6 was cotransfected with 4 μ g of an unrelated short hairpin construct or combined short hairpin (shDvl) constructs. Uncomplexed β -catenin levels were determined at 72 h posttransfection. A pool of antibodies against Dvl-1, Dvl-2, and Dvl-3 were used to detect endogenous Dvl proteins in 50 μ g of cell lysate. Tubulin levels served as a loading control. (e) Activation of TOPFLASH TCF luciferase reporter by Wnt5a/Dkk2C through multiple frizzled/LRP6 coreceptors. The expression of transfected proteins in panels a, b, c, and d were detected with anti-Flag antibody.

naling. To test whether the inability of Wnt5a to functionally interact with LRP6 was its only defect, we generated a chimeric ligand by tandemly linking Wnt5a and Dkk2C, reasoning that the Dkk2C moiety might provide the necessary link to LRP6 signaling. Figure 3a (left panel) shows that the Wnt5a/Dkk2C chimera alone strikingly increased β -catenin stabilization to a level comparable to that observed with Wnt3a, whereas the control fusion protein Wnt5a/green fluorescent protein, like

Wnt5a, was inactive in inducing β -catenin stabilization. As a further control, a Wnt3a/Dkk2C chimera acted similarly to Wnt5a/Dkk2C or Wnt3a in increasing β -catenin stabilization. Moreover, coexpression of LRP6 further increased uncomplexed β -catenin in response to Wnt5a/Dkk2C comparably to Wnt3a (Fig. 3a, right panel). These results indicate that Wnt5a/Dkk2C was fully functional in transducing canonical signaling.

Most Wnt proteins remain tightly cell associated (36, 39),

and we did not detect Wnt5a and Wnt5a/Dkk2C in conditioned medium of 293T cells following transient expression of these two proteins. To assess whether the Dkk2C moiety affects the secretion of the Wnt5a/Dkk2C chimera, cell surface biotinylation was performed. Figure 3b shows that both Wnt5a and Wnt5a/Dkk2C were secreted at similar levels, which excludes the possibility that its more efficient secretion was responsible for canonical signaling by Wnt5a/Dkk2C but not Wnt5a. FRP and LRP6 Δ C mimic the ligand binding domains of frizzled and LRP6, respectively. Moreover, each has been reported to act as a dominant negative to inhibit canonical Wnt signaling (2, 46). Figure 3c (upper panel) shows that cotransfection with either FRP or LRP6 Δ C markedly antagonized the ability of Wnt5a/Dkk2C to activate the β -catenin signaling. Furthermore, RNA interference using a short hairpin RNA oligonucleotide against endogenous LRP6 receptors significantly inhibited the β -catenin stabilization by the Wnt5a/Dkk2C chimera (Fig. 3c, lower panel). All these findings strongly imply that the canonical signaling by the Wnt5a/Dkk2C chimeric ligand required both endogenous frizzled and LRP6 receptors, both of which are known positive components in transducing canonical Wnt signaling, and argue against the possibility that this chimeric ligand acts to block an unknown inhibitory pathway.

Dishevelled (Dvl) is an essential component downstream of frizzled receptors in both Wnt/ β -catenin and Wnt/planar cell polarity pathways (34), and Wnt5a/frizzled has been reported to induce membrane translocation and phosphorylation of Dvl (9, 14). To further demonstrate that Wnt5a must functionally interact with endogenous frizzled(s), we used RNA interference to knock down expression of endogenous Dvl proteins. By this approach, endogenous Dvl protein levels were greatly reduced, as were the levels of stabilized β -catenin induced by Wnt5a/Dkk2C and Wnt3a (Fig. 3d). In contrast, Dvl RNA interference had no obvious effect on stabilized β -catenin induced by Δ N-LRP6, which lacks the LRP6 extracellular domain and is constitutively active independent of frizzled or Wnt (28, 47). Thus, Dvl is required for endogenous canonical signaling by Wnt5a/Dkk2C and Wnt3a. These results support the conclusion that Wnt5a has the innate capacity to function through endogenous frizzled(s) in a manner similar to that of a representative canonical Wnt, Wnt3a. To extend these findings, we assessed which frizzled(s) could cooperate with Wnt5a/Dkk2C by using the TOPFLASH TCF reporter assay. Figure 3e shows that the Wnt5a/Dkk2C signaling was enhanced to various extents by exogenously expressed frizzleds, with the exception of fz6 and fz9, in a pattern similar to that of Wnt3a, whereas Wnt5a alone did not cooperate with any of the frizzleds analyzed.

Activation of canonical Wnt signaling requires ligand coupling of an endogenous frizzled/LRP coreceptor complex. Canonical Wnt1 has been shown *in vitro* to bind to the extracellular domains of both frizzled and LRP6 receptors independently, leading to the formation of a coreceptor complex (46). However, it has not been established *in vivo* whether canonical Wnts act by recruiting the endogenous coreceptors or by activating each coreceptor independently with signals converging downstream to stabilize β -catenin. Evidence that Dkks interacted with LRP6 but not frizzled (4, 31, 42), while Wnt5a interacted with frizzled but not LRP6 (Fig. 1 and 2), made it possible to test which of these receptor activation

FIG. 4. Activation of canonical Wnt signaling requires ligand-induced coupling of endogenous frizzled and LRP6 coreceptors. (a) β -catenin stabilization by the Wnt5a/Dkk2C chimera but not by Wnt5a and Dkk2C together. Each ligand construct as indicated was transfected in 293T cells at 0.5 or 2 μ g. Uncomplexed β -catenin levels were measured after 48 h posttransfection. The anti-Flag antibody (α -Flag) was utilized to detect transfected proteins. α - β -cat, anti- β -catenin antibody; Vec, vector. (b) Paracrine TCF reporter assay. Target 293T cells transfected with Topglow TCF reporter (500 ng) and pRL-CMV (5 ng) were cocultured with 293T effector cells transfected with 2 μ g of each empty vector, Wnt5a, Dkk2C, Wnt5a plus Dkk2C, or Wnt5a/Dkk2C. Luciferase activities were measured 48 h later. RLU, relative luciferase units.

mechanisms was operational in cells under physiologic conditions. In the first model, Wnt5a and Dkk2C added together would not be expected to activate canonical signaling, while according to the second model, they should. Under conditions in which the Wnt5a/Dkk2C chimera activated canonical signaling through endogenous receptors in 293T cells, as evidenced by the dramatic increase in uncomplexed β -catenin elicited (Fig. 4a), Wnt5a and Dkk2C added together failed to induce β -catenin stabilization. Similarly, in a paracrine TCF reporter assay, expression of the Wnt5a/Dkk2C chimera by the effector cells activated TCF signaling in target cells, whereas neither Wnt5a or Dkk2C alone, or together, stimulated TCF reporter activity (Fig. 4b). These results strongly argue that efficient activation of endogenous canonical Wnt signaling must involve ligand recruitment of both frizzled and LRP into close proximity within the same coreceptor complex, rather than signaling independently through each receptor.

DISCUSSION

The identification of two types of receptors, frizzled and LRP5/6, as Wnt coreceptors has led to important biochemical insights into the roles of these receptors in canonical Wnt signaling. Frizzled is thought to have a signaling role because mutations in its intracellular region inactivate frizzled in transducing Wnt/ β -catenin signaling (50). Dvl protein has been defined as a key component downstream of frizzled through both genetic and biochemical analysis (1, 6, 22, 58). Axin, the key scaffold protein essential for β -catenin phosphorylation by GSK3 β (21), is another intracellular component in the Wnt/ β -catenin pathway connecting to the membrane receptors. It has been shown that Axin is recruited to the phosphorylated LRP5/6 receptors upon Wnt treatment (28, 32, 47). Moreover, Δ N-LRP5 and Δ N-LRP6 mutants lacking the extracellular domain are constitutively active and interact with Axin independent of Wnt and frizzled (28, 32, 47). All these findings imply that independent signaling events can be initiated from frizzled and LRP5/6 receptors upon Wnt triggering.

Two different models have been proposed regarding the synergistic cooperation between frizzled and LRP5/6 receptors in canonical Wnt signaling (16). The first model suggests that Wnt induces a frizzled/LRP complex which corecruits Dvl and Axin into the coreceptor complex via frizzled-Dvl and LRP5/6-Axin interactions. The second model doesn't necessarily require frizzled and LRP5/6 to form a coreceptor complex. Instead, frizzled-Dvl-Axin and LRP5/6-Axin would represent two parallel and independent branches which converge downstream to activate the β -catenin signaling under physiological conditions. Recent studies using exogenously expressed chimeric receptor constructs between *Drosophila* fz2 and the cytoplasmic domain of *arrow* (48), or the Hfz5 and LRP6 cytoplasmic domain (10), demonstrated that chimeric receptors mimicking the frizzled/LRP coreceptor complex are constitutively active in canonical signaling in support of the first model. However, those studies could not exclude that overexpression of receptor constructs may signal differently than ligand-triggered endogenous receptors. For instance, Dvl can be translocated to the plasmic membrane and be phosphorylated by overexpression of frizzleds (1, 6, 55), whereas under physiological conditions, this event occurs only upon Wnt treatment (60). Moreover, overexpression of Δ N-LRP6 constitutively activates β -catenin signaling independently of Wnt, frizzled, and Dvl, while endogenous Wnt signaling requires all these components.

The present unavailability of immunological reagents makes it impossible to directly measure the endogenous coreceptor complex of frizzled and LRP in vivo upon Wnt treatment. Thus, to establish the in vivo receptor activation mechanism, we employed a simple chimeric ligand strategy by fusing a noncanonical Wnt, which binds to frizzleds, with part of the Dkk2 ligand, which interacts with LRP receptors (7). In principle, the resulting chimeric ligand should be able to recruit both frizzled and LRP coreceptors into the same complex through each receptor binding moiety. Our results showed that Wnt5a and Dkk2C were independently or together unable to activate the β -catenin signaling. Only when these two moieties were fused together was canonical signaling efficiently activated. Furthermore, the canonical signaling by the Wnt5a/Dkk2C chimera was significantly inhibited by the dominant

negative of both frizzled and LRP receptors as well as by LRP6 small interfering RNA. All these results strongly argue that the activation of endogenous canonical Wnt signaling requires ligand coupling of both frizzled and LRP coreceptors in the same receptor complex rather than Wnt triggering each receptor independently.

A third Wnt coreceptor, Ryk, which belongs to the atypical receptor tyrosine kinase family and contains the Wnt inhibitory factor domain for Wnt binding, has been recently identified (29). This receptor is conserved in *Drosophila* (*Derailed*) (61) and in *Caenorhabditis elegans* (lin-18) (18) and may mediate a parallel Wnt signaling independent of frizzled (18). Whether or not the activation of canonical signaling through Ryk requires Wnt coupling of Ryk and LRP remains to be determined. Nonetheless, this coreceptor seems to be required for Wnt signaling in the development of certain tissues.

Previous studies have indicated that the *Xenopus* Wnt5a can induce axis duplication in *Xenopus* embryos through exogenous Hfz5 and LRP receptors (15, 46). The basis for the differences between those studies and our results, whether experimental or cell context dependent, remains to be determined. One possible explanation could be that the affinity of Wnt5a to LRP receptors is much lower than that of canonical Wnts like Wnt1, or Wnt3a, which limits detection of the activity of Wnt5a in mammalian cell cultures but not in *Xenopus* embryo assay with exogenously expressed receptors. Nonetheless, the coreceptor complex activation mechanism, which we demonstrate here for canonical Wnt signaling, functionally distinguishes Wnt5a, a prototype noncanonical Wnt, from canonical Wnts under conditions of physiological receptor expression. Although the physical interactions between Wnt5a and LRP5/6 receptors remain to be determined, our results indicate that Wnt5a is unable to functionally activate LRP in 293T cells and that this excludes Wnt5a from the canonical Wnt class. Conversely, the acquisition of canonical signaling by the noncanonical Wnt5a through its fusion to another LRP6 binding moiety, Dkk2C, unmasked its inherent ability to functionally interact with multiple frizzleds to signal through Dvl in a manner similar to that of canonical Wnts. Thus, the same frizzled receptor can be switched between distinct signaling pathways depending on the differential recruitment of the LRP coreceptor by members of the Wnt ligand family.

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