Wnt signals across the plasma membrane to activate the β-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP

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Summary

Wnt-induced signaling via β-catenin plays crucial roles in animal development and tumorigenesis. Both a seven-transmembrane protein in the Frizzled family and a single transmembrane protein in the LRP family (LDL-receptor-related protein 5/6 or Arrow) are essential for efficiently transducing a signal from Wnt, an extracellular ligand, to an intracellular pathway that stabilizes β-catenin by interfering with its rate of destruction. However, the molecular mechanism by which these two types of membrane receptors synergize to transmit the Wnt signal is not known. We have used mutant and chimeric forms of Frizzled, LRP and Wnt proteins, small inhibitory RNAs, and assays for β-catenin-mediated signaling and protein localization in *Drosophila* S2 cells and mammalian 293 cells to study transmission of a Wnt signal across the plasma membrane. Our findings are consistent with a mechanism by which Wnt protein binds to the extracellular domains of both LRP and Frizzled receptors, forming membrane-associated hetero-oligomers that interact with both Disheveled (via the intracellular portions of Frizzled) and Axin (via the intracellular domain of LRP). This model takes into account several observations reported here: the identification of intracellular residues of Frizzled required for β-catenin signaling and for recruitment of Dvl to the plasma membrane; evidence that Wnt3A binds to the ectodomains of LRP and Frizzled; and demonstrations that a requirement for Wnt ligand can be abrogated by chimeric receptors that allow formation of Frizzled-LRP hetero-oligomers. In addition, the β-catenin signaling mediated by ectopic expression of LRP is not dependent on Disheveled or Wnt, but can also be augmented by oligomerization of LRP receptors.

Key words: Wnt, LDL-receptor-related proteins 5 and 6, Frizzled, *Drosophila*

Introduction

Wnt proteins comprise a family of secreted glycoproteins that play diverse roles during development, such as cell proliferation, cell polarity and cell fate determination (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). Genetic experiments in *Drosophila* and biochemical studies in *Xenopus* and mammalian cells have established a framework for the Wnt signaling pathway (Peifer and Polakis, 2000). In cells that do not receive a Wnt signal, cytoplasmic β-catenin is associated with a multiprotein β-catenin destruction complex that contains several proteins, including Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3 (GSK3). In this complex, β-catenin is constitutively phosphorylated and degraded by the ubiquitin-proteasome pathway. The Wnt signal is received by two membrane receptors, Frizzled and LRP (LRP5/6, Arrow). Through an unknown mechanism that involves Dishevelled (Dvl), Wnt induces destabilization of the β-catenin destruction complex and stabilization of β-catenin. Accumulated β-catenin then enters the nucleus and, together with the TCF family of transcription factors, regulates transcription. Inappropriate activation of Wnt/β-catenin signaling can lead to cancer (Polakis, 2000).

Frizzled receptors are a family of seven-transmembrane proteins, which interact with Wnt proteins through their N-terminal extracellular cysteine-rich domain (CRD) (Bhanot et al., 1996; Hsieh et al., 1999). There are three major pathways downstream of Frizzled: the canonical Wnt/β-catenin pathway, the planar polarity pathway and the Wnt-Ca^{2+} pathway. It is unclear whether Frizzled receptors are directly coupled to the same set of downstream signaling proteins in these distinct signaling pathways. Like Wnt proteins, Frizzled receptors have been grouped into two classes: one class is thought to stimulate the β-catenin pathway and the other class to stimulate the Ca^{2+} pathway (Miller et al., 1999a). The mechanism by which Frizzled activates the Wnt/β-catenin pathway is still uncertain, although a link with heterotrimeric G proteins has been proposed (Liu et al., 2001). Dvl is, at present, the only known component closely downstream of Frizzled and it is involved in all three pathways downstream of Frizzled.

LRP co-receptors (LRP5/6, Arrow) are single transmembrane proteins that comprise a subfamily of LDL-

receptor related proteins and play an essential role in the canonical β-catenin pathway (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). The intracellular domains of LRP5 and Arrow have been shown to bind to Axin (Mao et al., 2001b; Tolwinski et al., 2003). Recent studies have demonstrated that Wnt induces membrane translocation and destabilization of Axin (Cliffe et al., 2003; Mao et al., 2001a; Tolwinski et al., 2003). Although it is widely accepted that Wnt binds to Frizzled, whether Wnt directly interacts with LRP is less certain (Tamai et al., 2000; Wu and Nusse, 2002). LRP has a large extracellular domain that contains four EGF repeats and three LDLR repeats. The role of the extracellular domain of LRP and the functional significance of the potential physical interaction between this domain and Wnt are unclear.

Both Frizzled and LRP receptors are essential for the signaling activity of Wnt. There are three potential mechanisms that could explain the interdependence between endogenous Frizzled and LRP in transmitting the Wnt signal. First, Wnt, Frizzled and LRP could form a single signaling complex, leading to the transmission of one signal. Second, Wnt could form two separate complexes with Frizzled and LRP, and generate signals that merge in downstream signaling cascades. Third, Wnt could primarily interact with one receptor, inducing activation of the other receptor through intracellular signaling. In the last two scenarios, proximity between Frizzled and LRP might be unnecessary and insufficient for signaling. Based on the finding that the extracellular domain of LRP6 binds to Wnt1 and associates with the extracellular domain of Frizzled in a Wnt-dependent manner, it has been proposed that Wnt triggers β-catenin signaling by bridging between Frizzled and LRP (Tamai et al., 2000). However, this model has not been rigorously tested.

In this study, we have analyzed the mechanisms by which Frizzled and LRP transmit a Wnt signal in cell culture systems. We have identified key amino acid residues of Frizzled essential for transducing the Wnt signal, and demonstrated that LRP signaling activity can be stimulated by oligomerization. We have provided several lines of evidence suggesting that Wnt activates β-catenin signaling by bringing Frizzled and LRP into proximity.

Materials and methods

Expression constructs

All *Drosophila* expression plasmids were made in the *Drosophila* expression construct pPac-PL. Wg, WgCS, rat Fz1, rat Fz2, human FZ5 and Armadillo ΔN (lacking amino acid residues 1-77) were cloned in a *Drosophila* expression vector under the control of the *Drosophila* actin promoter. Wg and WgCS were fused with an HA epitope at their C termini. Rat Fz1 and human FZ5 were fused with GFP at their C termini. Site-directed mutagenesis of Wg, rat Fz1 and human FZ5 was performed using two-step PCR. All constructs were verified by DNA sequencing. DFz2, LRP6, LRP6ΔN, LEF-1 and Renilla luciferase were cloned in pPac-PL and have been previously described (Schweizer and Varmus, 2003). The extracellular domain of human TRKC (residues 1-428) and the extracellular domain of human FZ5 (residues 1-235) were fused with a C-terminal fragment of human LRP6 (residues 1365-1612) to form TrkN-LRP6C and HFz5N-LRP6C. The intracellular domain of LRP6 (residues 1395-1612) was fused to the C terminus of human Fz5 to form HFz5-LRP6C. Wg and human NT3 were fused to the N terminus of human FZ5 to form Wg-HFz5 and NT3-HFz5, and a 24-amino acid linker $(GGGGSGGGT)_3$ was inserted between the N terminal Wg or NT3 fragment and the C-terminal human FZ5 fragment. Wg was fused to the N terminus of TrkN-LRP6C. GFP was fused to the C termini of HFz5-LRP6C, NT3-HFz5,and Wg-HFz5.

HFz5, LRP6, LRP6ΔEGF, LRP6ΔEGF-FKBP and LRP6ΔN were cloned into a mammalian expression vector under the control of the cytomegalovirus (CMV) promoter. GFP was fused to the C terminus of human FZ5 and YFP was fused to the C termini of LRP6 and LRP6ΔN. MESD (Hsieh et al., 2003), Myc-tagged Dsh and mFz8-CRD-IgG (Hsieh et al., 1999) were provided by Jen-Chih Hsieh. LRP6N-IgG and LDLR-IgG were provided by Xi He (Tamai et al., 2000). The LEF-1 reporter constructs were provided by Rudolf Grosschedl. TOP-FLASH reporter was provided by Hans Clevers.

Mammalian cell culture, transfection and luciferase assays

Human 293 cells and monkey COS7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Cells were transfected with Fugene 6 (Roche) according to the manufacturer's instructions. Luciferase assays were performed with the Dual Luciferase Assay kit (Promega) according to the manufacturer's instructions.

Drosophila S2 cell transfection and dsRNA experiments

Drosophila S2 cells were grown at room temperature in Schneider's *Drosophila* medium supplemented with 10% fetal bovine serum. Transfection of S2 cells and dsRNA experiments were performed as described (Cong et al., 2004). The following genes were targeted with dsRNA: *Drosophila* Arrow, DDBJ/EMBL/GenBank Accession Number NM_079998; Dsh, Accession Number L26974; *Drosophila* stimulatory G protein α subunit Gαs, Accession Number M23233. Gαs-dsRNA was used as a control in the RNA interference experiments.

Co-immunoprecipitation and immunoblotting assay

Co-immunoprecipitation of Frizzled and Dvl was performed as described (Chen et al., 2003). Co-immunoprecipitation of LRP6 and Axin was performed as described (Liu et al., 2003). Commercial antibodies used in this study include anti-GFP polyclonal antibodies (Clontech), anti-α-tubulin monoclonal antibodies (Sigma), anti-HA (HA.11) monoclonal antibodies (Covance), anti-β-catenin monoclonal antibodies (Transduction Laboratories) and anti-Myc (9E10) monoclonal antibodies (Santa Cruz).

Membrane biotinylation assay

Cells were washed twice with ice-cold PBS (pH 8.0), and suspended at a concentration of 1×10^7 cells/ml. Cells were incubated with 0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce) at room temperature for 30 minutes. Cells were washed once with 50 mM Tris (pH 8.0) and twice with ice-cold PBS to remove any remaining biotinylation reagents. Cells were then lysed with RIPA buffer and cell lysates were incubated with immobilized monomeric Avidin beads (Pierce) for 3 hours. Beads were washed four times with RIPA buffer, and the bound proteins were eluted with Laemmli sample buffer.

Cell fractionation assay

Cells were washed and scraped on ice into TBS (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 2 mM DTT, protease inhibitors). Cells were homogenized with 30 strokes in a dounce homogenizer, and the nuclei were removed by low speed centrifugation. The post-nuclear supernatants were spun at 100,000 ***g*** for 90 minutes at 4°C to generate a supernatant, or cytosolic, fraction and membrane-rich pellet fraction. Samples normalized for protein content were analyzed by SDS-PAGE.

Fluorescence microscopy

COS cells were transfected with Myc-tagged Dsh and GFP-tagged HFz5. The immunofluorescence experiment was performed as

described (Cong et al., 2003). 293 cells were transfected with LRP6-YFP and LRP6ΔN-YFP expression constructs. Thirty-six hours after transfection, cells were fixed with 2% paraformaldehyde. Cells were washed with PBS, mounted using Vetashield mounting medium and examined by laser scanning microscopy (Zeiss).

Liquid binding assay

Liquid binding assay was performed as previously described (Semenov et al., 2001; Tamai et al., 2000). All recombinant proteins were used as conditioned medium (CM). LRP6N-IgG, LDLR-IgG and mFz8CRD-IgG were produced in 293 cells via transient transfection. MESD was co-expressed with LRP6N-IgG to facilitate the secretion of LRP6N-IgG. Wnt3A and Wnt3A C77S-HA CM were collected from Rat-2 cells stably transfected with Wnt3A or Wnt3A C77S.

Results

Ligand-dependent activation of Frizzled receptors

Signaling activities of various Frizzled receptors in the β-catenin pathway were measured using a LEF-1 luciferase assay (Schweizer and Varmus, 2003). S2 cells contain relatively low levels of endogenous *Drosophila* Frizzled proteins (Bhanot et al., 1996; Schweizer and Varmus, 2003), and Wingless (Wg) modestly increased LEF-dependent transcription (Fig. 1). Ectopic expression of mammalian Frizzled receptors in S2 cells had minimal effects on LEF-dependent transcription (Fig. 1); however, the stimulatory effect of Wg was greatly enhanced by co-expression of *Drosophila* Frizzled 2 (Fz2) (Schweizer and Varmus, 2003), human frizzled 5 (FZ5) or Rat frizzled 1 (Fz1) (Fig. 1), consistent with the known function of these Frizzled receptors in β-catenin signaling (Bhat, 1998; He et al., 1997; Kennerdell and Carthew, 1998; Yang-Snyder et al., 1996). Treatment of S2 cells with double-stranded RNA (dsRNA) that inhibits production of Arrow, Dsh (*Drosophila* Dishevelled), or Armadillo (*Drosophila* β-catenin) significantly decreased the stimulatory effects of Wg and Frizzled on LEF-dependent transcription (Schweizer and Varmus, 2003).

Surprisingly, Rat Frizzled 2 (RFz2) also greatly increased Wg-induced LEF-dependent transcription (Fig. 1). Rat Fz1 belongs the class of Frizzled proteins that selectively activate the β-catenin pathway, whereas rat Fz2 has been assigned to the other class that selectively activate the Ca^{2+} pathway (Sheldahl et al., 1999; Slusarski et al., 1997; Yang-Snyder et al., 1996). It has been suggested that the signaling specificity of rat Fz1 and rat Fz2 is determined by their intracellular domains (Ahumada et al., 2002; Liu et al., 2001). Our data suggest that both rat Fz1 and rat Fz2 can initiate β-catenin signaling through interacting with cytoplasmic components of the β-catenin pathway, at least in S2 cells.

Identification of amino acid residues in the intracellular domains of Frizzled receptors essential for Wnt/β-catenin signaling

We systematically mutagenized the intracellular domains of rat Fz1 to identify critical amino acid residues for Wnt/β-catenin signaling using the S2 cell culture assay. Thirty rat Fz1 mutants were generated to make changes in all three intracellular loops and in the proximal membrane region of the C-terminal tail, a region known to be essential for Wnt signaling (Umbhauer et al., 2000), and two mutants were generated with changes in the extracellular loops. In each mutant, two neighboring amino acid residues were simultaneously changed to Ala. As expected, mutations in the extracellular loops did not affect the signaling activity of rat Fz1, while mutations in the intracellular domains had various effects (Fig. 2A). All rat Fz1 mutants were tagged with a GFP epitope at their C termini; immunoblotting demonstrated that the levels of mutant proteins were approximately equal (data not shown).

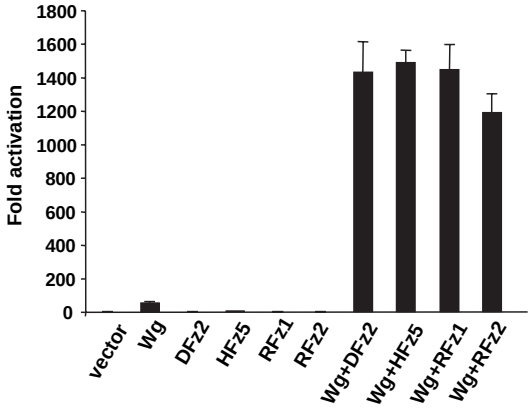

Fig. 1. Synergistic effects of Wg and Frizzled on LEF-dependent transcription. S2 cells were transfected with indicated plasmids and plasmids as described in the Materials and methods. Fold-activation values were measured relative to the levels of luciferase activity in cells transfected with an empty vector and normalized by *Renilla* luciferase activities. All experiments were carried out in triplicate and error bars represent standard deviation.

Next, we generated another 16 mutants covering the critical loop regions of rat Fz1 defined in the previous experiment, changing a single amino acid residue to Ala in each mutant. In this analysis, mutations at three residues, Arg340 in the first loop, and Leu524 and Leu527 in the third loop, severely affected the function of rat Fz1, whereas other mutations had only marginal or moderate effects (Fig. 2B and data not shown). Consistent with a previous report (Umbhauer et al., 2000), Lys619 in the Lys-Thr-x-x-x-Trp motif in the C-terminal tail of Frizzled is also essential for β-catenin signaling (Fig. 2B). Mutating Arg340, Leu524 and Lys619 to Ala did not affect the membrane localization of rat Fz1 proteins, as demonstrated by membrane biotinylation (Fig. 2B). The specificity of membrane biotinylation was demonstrated by using a cytoplasmic protein, Myc-tagged Dbt (doubletime), as a negative control (data not shown). Mutating Leu527 to Ala significantly decreased the amount of rat Fz1 on the plasma membrane (data not shown), so this mutant was excluded from further studies.

We then mutated Arg263, Leu443 and Lys525 of human FZ5, residues analogous to Arg 340, Leu524 and Lys619 of rat Fz1, and found that mutating these residues abolished the signaling activity of human FZ5 without affecting its membrane localization (Fig. 2C). All three residues are well conserved in all Frizzled receptors (data not shown).

The Trp residue in the essential Lys-Thr-x-x-x-Trp motif is conserved in most Frizzled receptors, but substituted by a Tyr residue in *Drosophila* Fz3 and mom-5 of *C. elegans*. As *Drosophila* Fz3 and mom-5 appear to be weak or defective Frizzled receptors (Rocheleau et al., 1997; Sato et al., 1999;

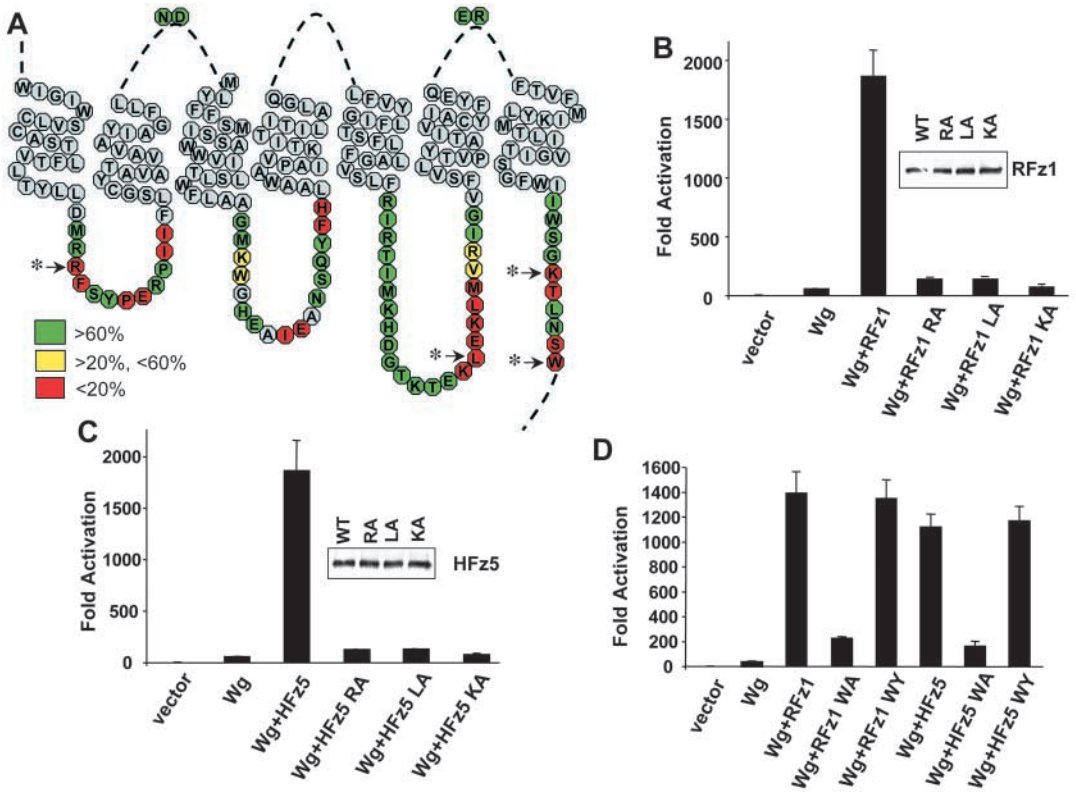

Fig. 2. Activation of Wnt/β-catenin signaling by rat Fz1 and human FZ5 point mutants. (A) Schematic representation of rat Fz1 double-alanine-scanning mutants. Amino acids residues of transmembrane segments, three intracellular loops and the N-terminal section of the intracellular tail were shown. In these rat Fz1 mutants, two neighboring amino acid were changed to Ala, and a GFP tag was fused to the C termini of all mutants. The majority of mutants cover the intracellular regions, while two mutants reside in the extracellular loops. The activities of wild-type and mutant rat Fz1 to stimulate LEF-dependent transcription were measured in S2 cells. The activities of mutants rat Fz1 relative to that of wild-type rat Fz1 are indicated by color. Amino acid residues essential for the signaling activity of rat Fz1 and human FZ5 and studied in detail in later experiments are indicated by asterisks. (B) Single point mutations abolish signaling activity of rat Fz1. Activities of rat Fz1, rat Fz1 R340A, rat Fz1 L524A and rat Fz1 K619A were measured in S2 cells. The membrane localization of wild-type and mutant rat Fz1 was examined by membrane biotinylation, precipitation with avidin-agarose beads and immunoblotting with anti-GFP antibodies (insert). (C) Single point mutations abolish signaling activity of human FZ5. Activities of human FZ5, human FZ5 R263A, human FZ5 L443A and human FZ5 K525A were measured in S2 cells. All human FZ5 constructs were fused with a GFP epitope at their C termini. The membrane localizations of wild-type and mutant human FZ5 were examined by the membrane biotinylation assay (insert). (D) The Trp residue in the Lys-Thr-x-x-x-Trp motif can be substituted by Tyr. The Trp residue in the Lys-Thr-x-x-x-Trp motif (W624 in rat Fz1 and W530 in human FZ5) was mutated to either Ala or Tyr, and activities of these mutants were measured in S2 cells.

Thorpe et al., 1997), it has been speculated that the Trp to Tyr substitution might be responsible for the defective signaling activities of *Drosophila* Fz3 and mom-5 (Umbhauer et al., 2000). We have found that mutating the corresponding Trp residue in rat Fz1 and human FZ5 to Ala significantly reduced the signaling activity of Frizzled receptors, but mutating it to Tyr had no significant effect (Fig. 2D). Therefore, replacement of Trp with Tyr at this position in *Drosophila* Fz3 and mom-5 is unlikely to be responsible for their deficiencies in Wnt signaling.

Membrane relocation of Dvl by wild-type and mutant Frizzled receptors

Overexpression of Frizzled induces membrane translocation of Dvl. We tested whether the residues of Frizzled identified as crucial for β-catenin signaling were also important for Frizzled-induced Dvl membrane translocation. Dsh was localized in a punctated pattern in the cytoplasm when expressed alone in COS cells, and co-expression of human FZ5 recruited Dsh to the plasma membrane (Fig. 3A). By contrast, the inactive human FZ5 mutants R253A, L443A and K525A were unable to translocate Dsh to the plasma membrane (Fig. 3A), although they were all present on the plasma membrane at levels similar to that of wild-type protein as determined by membrane biotinylation (data not shown).

We next tested whether the implicated residues are required to form a complex containing Frizzled and Dvl by performing a co-immunoprecipitation experiment. Although wild-type human FZ5 was found in immunoprecipitates containing Dsh, the three human FZ5 mutants were not (Fig. 3B). Taken together, these data suggest that mutations that disrupt the signaling activities of Frizzled in the β-catenin pathway also affect the formation of a membrane-associated complex that includes Frizzled and Dvl.

Activation of LRP6 signaling activity by inducible oligomerization

Overexpression of LRP without Wnt and Frizzled stimulates

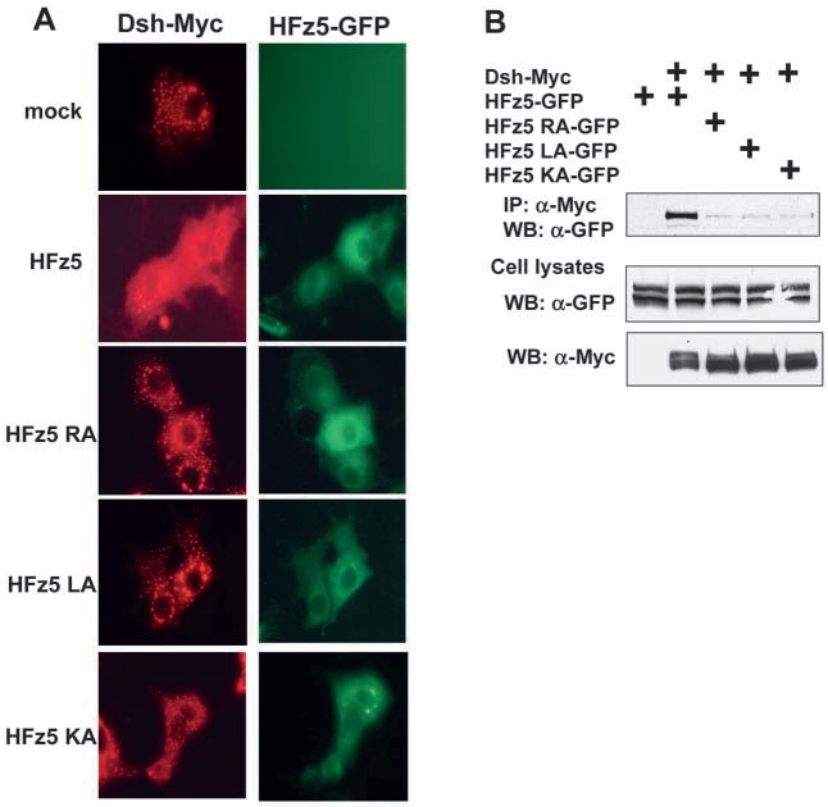

Fig. 3. Point mutations decrease the interaction between Dsh and HFz5. (A) Recruitment of Dsh to the plasma membrane by wild-type and mutant human FZ5. Myc-tagged Dsh and GFP-tagged human FZ5 were co-expressed in COS cells. Cells were stained by anti-Myc 9E10 monoclonal antibodies and Texas Red-conjugated goat anti-mouse secondary antibodies, and examined by fluorescence microscopy. (B) Physical interaction between Dsh and wild-type or mutant human FZ5. Myc-tagged Dsh and GFP-tagged human FZ5 were co-expressed in 293 cells. Cell lysates were immunoprecipitated with anti-Myc antibodies, and immunoprecipitates were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with anti-GFP antibodies (upper panel). Expression of Dsh and human FZ5 in total cell lysates was examined by immunoblotting with anti-Myc and anti-GFP antibodies (lower panels).

β-catenin signaling in various systems (Mao et al., 2001b; Schweizer and Varmus, 2003; Tamai et al., 2000), possibly by titrating endogenous Axin. As many receptors are activated by ligand-induced oligomerization (Weiss and Schlessinger, 1998), it is conceivable that ligand-induced oligomerization of LRP increases its signaling activity.

We tested this hypothesis by generating various LRP chimeric proteins. The FK506-binding protein, FKBP, has been engineered to serve as an inducible intracellular dimerization domain (Klemm et al., 1998). LRP6ΔEGF-FKBP was generated by fusing two copies of a modified FKBP domain (Ariad) to the C terminus of LRP6ΔEGF, an LRP6 N-terminal deletion mutant with all four EGF repeats removed (Fig. 4A) and its activity was tested in 293 cells using a LEF-luciferase reporter as readout. AP20187, a synthetic dimerizer (Clackson et al., 1998), significantly increased the signaling activity of LRP6ΔEGF-FKBP, but not that of LRP6ΔEGF (Fig. 4B). Furthermore, AP20187 increased LRP6ΔEGF-FKBP-induced, but not LRP6ΔEGF-induced, stabilization of β-catenin in 293 cells (Fig. 4C). These results suggest that oligomerization of LRP increases the signaling activity of LRP in the β-catenin pathway. This conclusion directly conflicts with a recent report by Liu et al. (Liu et al., 2003), as discussed later.

As Axin directly interacts with the intracellular domain of LRP (Mao et al., 2001b; Tolwinski et al., 2003), we tested whether oligomerization of LRP6 affects its binding to Axin in 293 cells. As shown in Fig. 4D, AP20187 did not affect the binding of LRP6ΔEGF to Axin, but clearly increased the interaction of LRP6ΔEGF-FKBP with Axin. These data suggest that oligomerization of LRP6 increases its signaling activity by enhancing recruitment of Axin.

Next, we tested the effect of oligomerization on the signaling activity of LRP6 in *Drosophila* S2 cells using a different strategy, involving an extracellular oligomerization signal. The extracellular domain of TrkC, a neurotrophin receptor, was fused N-terminal to the transmembrane domain of LRP6 to form TrkN-LRP6C (Fig. 4A); neurotrophin 3 (NT3), a specific ligand of TrkC, was used to induce oligomerization of TrkN-LRP6C. Treatment of *Drosophila* S2 cells with NT3 increased the signaling activity of TrkN-LRP6C by almost tenfold (Fig. 4E), but had no effect on the signaling activity of LRP6, LRP6ΔN and HFz5N-LRP6C, a chimeric protein with the N-terminal extracellular domain of human FZ5 fused upstream of the transmembrane domain of LRP6 (Fig. 4A). These data further strengthen the claim that the signaling activity of LRP can be increased by oligomerization.

We next tested whether Dvl is required for the signaling activity of LRP in these experiments. Although Dsh-dsRNA significantly decreased the signaling activity of Wg and Fz5 (Fig. 4F), it had no effect on the signaling activity of LRP6ΔN, consistent with previous findings (Li et al., 2002; Schweizer and Varmus, 2003). Importantly, Dsh-dsRNA also had no effect on the activity of TrkN-LRP6C, even in the presence of NT3. These results demonstrate that activation of β-catenin signaling by overexpression of LRP, with or without inducible oligomerization, does not involve Dvl. Presumably, overexpressed LRP stimulates β-catenin signaling by recruiting Axin to the plasma membrane and inactivating it, a process that does not require Dvl; oligomerized LRP has a higher affinity for Axin.

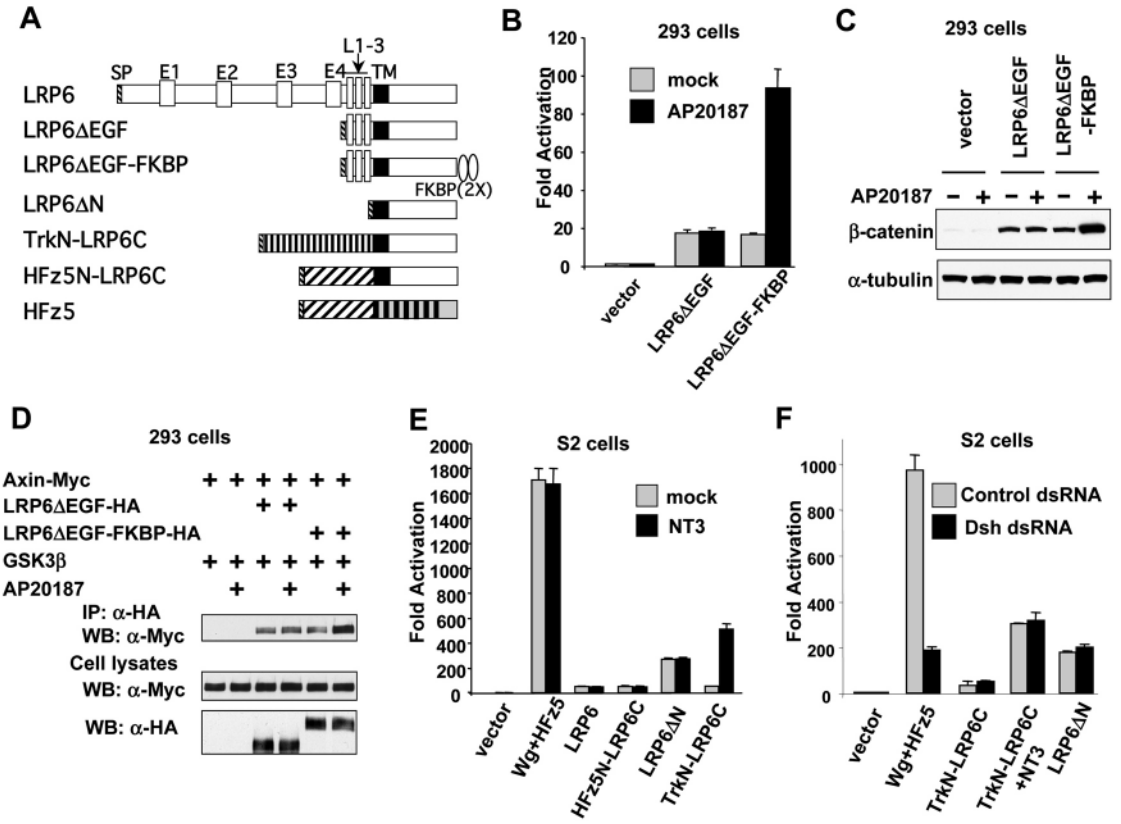

Fig. 4. Stimulation of the signaling activity of LRP6 by oligomerization. (A) Schematic representation of wild-type and mutant LRP6. Signal peptide (SP), EGF repeats (E1-E4), LDLR repeats (L1-3) and transmembrane domain (TM) are indicated. In LRP6ΔEGF, all EGF repeats were deleted. In LRP6ΔN, the whole extracellular domain was deleted. Two copies of FKBPv were tethered to the C terminus of LRP6ΔEGF to form LRP6ΔEGF-FKBP. The extracellular domain of LRP6 was replaced by the extracellular domain of TrkC or human FZ5 to form TrkN-LRP6C and HFz5N-LRP6C. (B) AP20187 increases the stimulatory effect of LRP6ΔEGF-FKBP on LEF-dependent transcription in 293 cells. 293 cells were transfected with the indicated plasmids and CMV-LEF1, CMV-*Renilla* luciferase and a LEF-luciferase reporter. Cells were either treated with vehicles or 50 nM AP20187 for 36 hours. The levels of luciferase activities were normalized for *Renilla* luciferase activities. (C) AP20187 increases LRP6ΔEGF-FKBP-induced β-catenin stabilization in 293 cells. 293 cells were transfected with the indicated plasmids and treated with vehicles or 50 nM AP20187 for 36 hours. Cells were subjected to subcellular fractionation, and cytosolic β-catenin was determined by immunoblotting. (D) AP20187 increases the binding of Axin to LRP6ΔEGF-FKBP during β-catenin stabilization in 293 cells. 293 cells were transfected with the indicated plasmids and treated with vehicles or 50 nM AP20187 for 36 hours. Proteins were immunoprecipitated from cell lysates with anti-HA antibodies, and immunoprecipitates were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with anti-Myc antibodies (upper panel). Expression of Axin and LRP6ΔEGF or LRP6ΔEGF-FKBP in total cell lysates was examined by immunoblotting with anti-Myc and anti-HA antibodies (lower panels). (E) NT3 increases the signaling activity of TrkN-LRP6C in S2 cells. S2 cells were transfected with the indicated plasmids and treated with 200 ng/ml NT-3 (Upstate) for 48 hours. Cells were lysed and the luciferase activities were normalized for *Renilla* luciferase activity. (F) The signaling activity of LRP6, with or without inducible oligomerization, is Dsh independent. S2 cells were treated with dsRNAs, transfected with the indicated effector plasmids and treated with 200 ng/ml NT3 for 48 hours. Cells were lysed and luciferase activities were measured.

The extracellular domain of LRP6 plays a positive role in Wnt signaling, and can be replaced by the N-terminal extracellular domain of human FZ5

Both LRP and Frizzled are required to transduce a Wnt signal. Could this occur by formation of hetero-oligomers and, if so, how? It is also uncertain whether all Wnt proteins bind to the extracellular domain of LRP and, if so, whether such a binding is functionally significant. It has been suggested that the extracellular domain, specifically the region containing the first two EGF repeats, plays an important role in transducing the Wnt signal (Mao et al., 2001a).

The extracellular domain of LRP might play a positive role and actively transmit the Wnt signal. Alternatively, as it has recently been suggested (Liu et al., 2003), the extracellular domain could play a negative role by restraining the signaling activity of LRP in the absence of Wnt; binding to Wnt might then release the inhibitory effect. Consistent with the second possibility, an LRP mutant lacking the whole extracellular domain appears to be hyperactive and does not synergize with Wnt (Liu et al., 2003; Mao et al., 2001a; Mao et al., 2001b; Schweizer and Varmus, 2003).

We tested the signaling activity of LRP6 and LRP6ΔN in 293 cells using the TOP-FLASH reporter. Consistent with previous observations, LRP6ΔN appeared to be much more active than LRP6 (Fig. 5B). However, a close examination of the subcellular localizations of these two proteins tagged at their C termini with YFP revealed that LRP6ΔN was mostly localized on the plasma membrane, while LRP6 was mostly

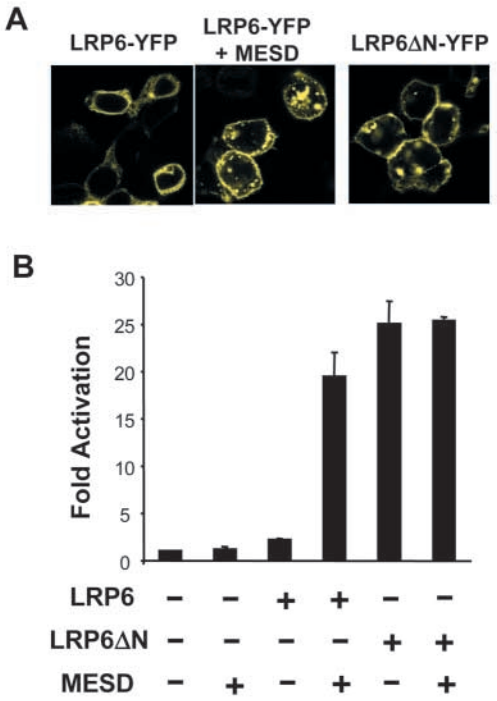

Fig. 5. The MESD chaperone protein increases the membrane localization and signaling activity of LRP6. (A) MESD increases the membrane localization of LRP6. LRP6 and LRP6ΔN were fused with YFP at their C termini, and expressed in 293 cells with or without co-expression of MESD. The subcellular localization of wild-type and mutant LRP6 was examined by confocal fluorescence microscopy. (B) MESD increases the signaling activity of LRP6. 293 cells were transfected with the indicated plasmids together with TOP-FLASH and CMV-*Renilla*. The luciferase activities were normalized for *Renilla* luciferase activities.

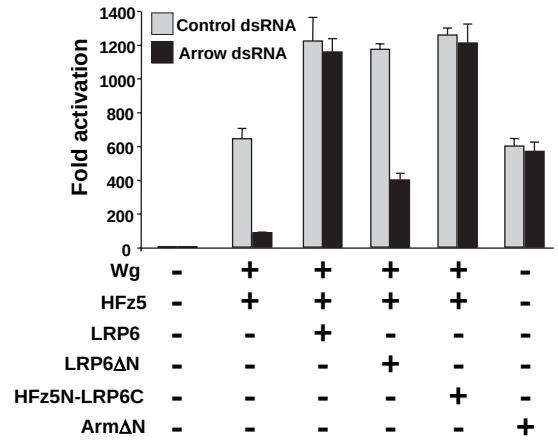

Fig. 6. Functional substitution of the extracellular domain of LRP6 with the extracellular domain of human FZ5. S2 cells were treated with the indicated dsRNAs and transfected with the indicated effector plasmid together with LEF-1 reporter plasmids. Forty-eight hours after transfection, cells were lysed and luciferase activities were measured.

localized in the perinuclear region, presumably in the endoplasmic reticulum (Fig. 5A). This observation is consistent with recent findings that correct folding and membrane translocation of LRP proteins require MESD (Boca in *Drosophila*), a chaperone protein found in the endoplasmic reticulum (Culi and Mann, 2003; Hsieh et al., 2003). Co-expression of MESD increased the membrane localization of LRP6 (Fig. 5A). Importantly, the signaling activities of LRP6 and LRP6ΔN became comparable upon co-expression of MESD (Fig. 5B). Therefore, the apparent lower activity of the full-length LRP6 is most likely due to its inefficient transport to the plasma membrane, and the extracellular domain of LRP6 does not play a negative role.

We have previously demonstrated that the extracellular domain of LRP plays a positive role in transducing the Wnt signal by showing that full-length LRP, but not N-terminally truncated LRP, is able to complement endogenous Arrow in S2 cells (Schweizer and Varmus, 2003). Whether Wnt protein directly interacts with LRP receptors is still controversial (Tamai et al., 2000; Wu and Nusse, 2002). Nevertheless, we reasoned that if the main function of the extracellular domain of LRP is to interact with Wnt, it might be functionally substituted by the N-terminal extracellular domain of Frizzled, which is known to bind to Wnt. XWnt8 present in conditioned medium is found in a large complex, as judged by gel filtration, and promotes dimerization of tagged Frizzled CRDs (Dann et al., 2001), suggesting that Wnt forms dimers or higher oligomers.

HFz5N-LRP6C was generated by fusing the extracellular domain of human FZ5 N-terminal to the transmembrane domain of LRP6 (Fig. 4A), and tested for its ability to complement endogenous Arrow in S2 cells. Consistent with our previous results (Schweizer and Varmus, 2003), full-length LRP, but not the N-terminally truncated LRP6, complemented endogenous Arrow to transduce the Wnt signal (Fig. 6). Significantly, HFz5N-LRP6C, like full-length LRP6, also complemented endogenous Arrow in this assay (Fig. 6). No synergy between HFz5N-LRP6C and human FZ5 was observed when Wg was not co-expressed (data not shown). The inability of LRP6ΔN to complement endogenous Arrow is not due to its lower signaling activity, as LRP6ΔN has a significantly higher activity than LRP6 and HFz5N-LRP6C when expressed alone (Fig. 4). Taken together, these data suggest that the extracellular domain of LRP plays a positive role in transducing the Wnt signal, possibly by binding to Wnt.

Hyperactivity of a Frizzled-LRP fusion protein

If Wnt binds to both LRP and Frizzled, there are at least two models that could explain how the Wnt signal is transmitted. As previously proposed (Tamai et al., 2000), it is possible that Wnt recruits LRP and Frizzled into the same complex, and a signal is transmitted when the two receptors are in proximity. Alternatively, Wnt could bind to and activate LRP and Frizzled at different locations on the cell surface, and signals generated independently by the two receptor complexes could synergize downstream.

A recent study by Tolwinski et al. supports the first model by showing that a Frizzled-LRP chimeric protein with the intracellular domain of Arrow fused to the C terminus of *Drosophila* Fz2 is more active than Arrow or *Drosophila* Fz2 and does not require endogenous Wg (Tolwinski et al., 2003). However, it is not clear from their study whether *Drosophila* Fz2 plays an active signaling role in the chimera. As

overexpression of LRP induces ligand-independent signaling in *Xenopus* embryos and cultured cells (Mao et al., 2001a; Mao et al., 2001b; Schweizer and Varmus, 2003; Tamai et al., 2000), the ligand-independent signaling of the *Drosophila* Fz2-Arrow chimera could result from enhanced stability of Arrow or increased membrane levels of Arrow. Furthermore, fusing *Drosophila* Fz2 to Arrow will increase oligomerization and therefore increase the signaling activity of Arrow, as Frizzled receptors are known to form constitutive homo-oligomers (Kaykas et al., 2004).

To explore these possibilities, we fused the intracellular domain of LRP6 to the C terminus of human FZ5, forming HFz5-LRP6C, then tested the signaling activity of this chimera in S2 cells. HFz5-LRP6C was much more active than human FZ5 or LRP6ΔN (Fig. 7), consistent with the previous findings (Tolwinski et al., 2003). Furthermore, mutating three residues essential for human FZ5 function, Arg263, Leu443 and Lys525 of HFz5-LRP6C, decreased the signaling activity of the chimera to a level similar to that of LRP6 alone, suggesting that human FZ5 is essential for signaling by the chimera. Mutating these residues in HFz5-LRP6C did not affect the membrane concentration of these proteins, as demonstrated by membrane biotinylation (Fig. 7). Importantly, the signaling activity of HFz5-LRP6C was Dsh-dependent, while the residual activity of mutated HFz5-LRP6C was Dsh independent (Fig. 7). Since the signaling activity of LRP6 is strictly Dsh-independent (Fig. 4), the residual activity of mutated HFz5-LRP6C is most probably contributed by only the LRP6 part of the chimera, and the higher signaling activity of HFz5-LRP6C could not result from enhanced oligomerization of LRP6. Taken together, these data suggest that Frizzled and LRP synergize when the two proteins are in proximity.

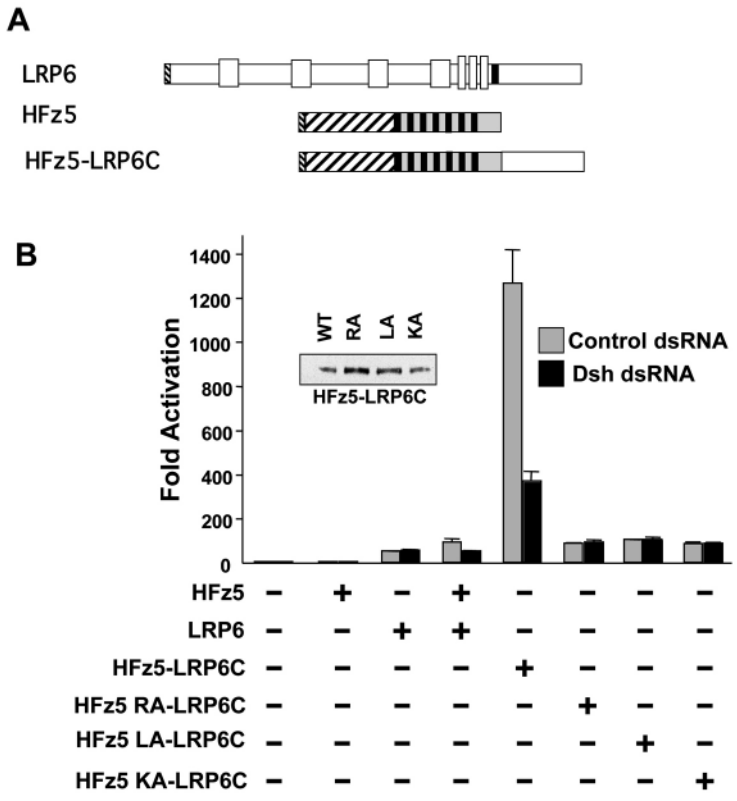

Fig. 7. Strong synergy between human FZ5 and LRP6 in Wnt/β-catenin signaling by fusing the intracellular domain of LRP6 to human FZ5. (A) Schematic representation of human FZ5, LRP6 and the HFz5-LRP6 chimera. The intracellular domain of LRP6 was fused to the C terminus of human FZ5 to form HFz5-LRP6C. The C terminus of HFz5-LRP6C was fused with GFP. (B) Hyperactivity of HFz5-LRP6C. S2 cells were treated with control or Dsh-dsRNA and transfected with the indicated plasmids. As control, Arg263, Leu443 or Lys525 of human FZ5 was mutated in HFz5-LRP6C. Membrane expressions of HFz5-LRP6C and its mutants were determined by the membrane biotinylation assay (insert).

Enhanced Wnt signaling activity with dimerization of human FZ5 and LRP6

In the experiment shown in Fig. 7, we could not confirm that the LRP6 region of HFz5-LRP6C played an active signaling role. Therefore, it remains possible that tethering the intracellular domain of LRP6 to human FZ5 changes the conformation of human FZ5, rendering it hyperactive. To exclude this possibility, we generated additional chimeras.

As NT3 is the ligand for TrkC, we fused NT3 to the N terminus of human FZ5 via a Gly-rich sequence linker to form NT3-HFz5 (Fig. 8A). Like human FZ5, NT3-HFz5 had no signaling activity by itself, but strongly synergized with Wg (Fig. 8B). Importantly, NT3-HFz5 strongly synergized with TrkN-LRP6C, which contains the extracellular domain of TrkC, but not with LRP6ΔN (Fig. 8B). Likewise, TrkN-LRP6C did not synergize with human FZ5 or with NT3-HFz5 K535A (Fig. 8B). Furthermore, the synergistic activity of TrkN-LRP6C and NT3-Fz5 was Dsh dependent (data not shown), implying that the human FZ5 part of the NT3-HFz5 chimera played an active signaling role. These experiments further strengthen the argument that bringing LRP and human FZ5 into proximity significantly increases their signaling activity via the β-catenin signaling pathway.

Requirement of free Wnt proteins can be bypassed by fusing Wnt to either Frizzled or LRP

If a major function of Wnt is to bind to both LRP and Frizzled to form a signaling complex, the requirement for free Wnt proteins might be by-passed by fusing Wnt to either of the two receptors.

It has previously been shown that fusing XWnt8 to the N terminus of human FZ5 results in a hyperactive molecule (Holmen et al., 2002). We generated a similar chimera by fusing Wg to the N terminus of human FZ5 (Fig. 9A); as anticipated, this chimera is hyperactive in S2 cells (Fig. 9B). The signaling activity of Wg-Fz5, like that of co-expression of Wg and human FZ5, was found to be dependent on both Arrow and Dsh (Fig. 9B).

We next tested whether the requirement for wild-type Wnt protein could be by-passed by fusing Wnt to LRP chimers. When Wg was fused to the N terminus of TrkN-LRP6C, with the Trk region serving as a linker (Fig. 9A), the product, Wg-TrkN-LRP6C, was found to be much more active in the β-catenin reporter assay in S2 cells than were TrkN-LRP6C or free Wg plus TrkN-LRP6C (Fig. 9D). Importantly, the activity of Wg-TrkN-LRP6C was Dsh dependent (Fig. 9D), indicating

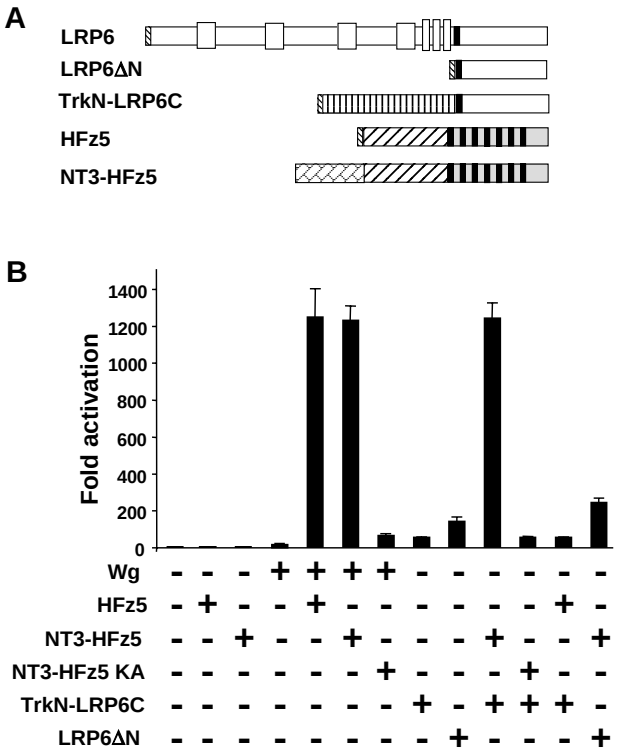

Fig. 8. Strong synergy between TrkN-LRP6C and NT3-HFz5 in Wnt/β-catenin signaling. (A) Schematic representation of human FZ5, LRP6 and the NT3-HFz5 chimera. NT3 was fused to the N terminus of human FZ5 to form NT3-HFz5. (B) Synergetic effect of TrkN-LRP6C and NT3-HFz5 on LEF-dependent transcription. S2 cells were transfected with the indicated plasmids. Luciferase activities were measured 48 hours after transfection.

involvement of Frizzled. As the signaling activity of various forms of LRP6 was Dsh independent (Fig. 4; data not shown), hyperactivity of Wg-TrkN-LRP6C was not simply a result of enhanced expression or oligomerization of the LRP6 domain. Presumably, the Wg fragment of the Wg-TrkN-LRP6C chimera interacts with a Frizzled protein endogenous to S2 cells (Schweizer and Varmus, 2003).

Taken together, the above experiments demonstrate that the requirement for native Wnt protein for β-catenin-mediated signaling can be by-passed by fusing Wnt to either Frizzled or LRP. This is consistent with the Wnt-induced dimerization model.

Potential roles for Wnt palmitoylation revealed by Wnt-Frizzled and Wnt-LRP chimeras

Wnt proteins are palmitoylated at the conserved Cys residue closest to the N terminus in the Wnt family of proteins, and this modification is required for the function of Wnt proteins (Willert et al., 2003). It is not known, however, why palmitoylation is required for Wnt function. Although palmitoylation does not appear to be required for secretion of at least some Wnt proteins, it might facilitate the ability of Wnt to interact with Frizzled, LRP, or some unknown molecules; alternatively, the palmitate group may promote membrane localization of Wnt.

A model of Wnt-dependent dimerization of receptors predicts that the main function of the Wg region of the Wg-HFz5 chimera is to interact with LRP, while the primary role of the Wg region of the Wg-TrkN-LRP6C chimera is to interact with Frizzled. Thus, these chimeric receptors provided an opportunity to test the role of palmitoylation in the interplay of Wnt with its two putative receptors.

We first generated a Wg mutant (WgCS), with the conserved potential palmitoylation site Cys93 mutated to Ser, and tested its signaling activity in S2 cells. As shown in Fig. 9C, WgCS was expressed at a similar level as Wg (data not shown), but completely lacked signaling activity, confirming the importance of palmitoylation. Mutating Cys93 to Ser in Wg-Fz5 (WgCS-HFz5) also severely affected the signaling activity of Wg-Fz5 without altering its membrane localization (Fig. 9C). Assuming that the primary role of Wg in the Wg-HFz5 chimera is to interact with Arrow, these results imply that the palmitate modification of Wnt proteins is important for the interaction between Wnt and LRP.

We next mutated Cys93 to Ser in Wg-TrkN-LRP6C to make WgCS-TrkN-LRP6C, and tested its signaling activity in S2 cells. Although WgCS-TrkN-LRP6C and Wg-TrkN-LRP6C were present at similar levels in the membrane, the signaling activity of WgCS-TrkN-LRP6C is significantly lower (Fig. 9D), suggesting that palmitoylation of Wnt is important for its binding to Frizzled. Consistent with this result, the WgCS-HFz5 mutant was significantly more active than human FZ5 plus WgCS, although the expression level of WgCS-HFz5 was lower than that of human FZ5 (Fig. 9C), so the signaling activity of WgCS can be partially rescued by fusing WgCS to human FZ5. This is consistent with the hypothesis that palmitoylation of Wnt might be important for the binding of Wnt to Frizzled. However, it is also possible that fusion of human FZ5 to WgCS enhances secretion of WgCS (Nusse, 2003) or tethers WgCS to the plasma membrane.

Physical interactions between Wnt and Frizzled or LRP

We have provided evidence to suggest that the major function of the extracellular domain of LRP is to bind to Wnt, and that Wnt most probably triggers the β-catenin pathway by forming Frizzled-LRP oligomers. However, the binding of Wnt to LRP is less well established than the binding of Wnt to Frizzled (Tamai et al., 2000; Wu and Nusse, 2002). We therefore examined the binding between Wnt and LRP using a liquid binding assay in which conditioned medium (CM) from Rat2 cells overexpressing HA-tagged Wnt3A was mixed with CM from 293 cells overexpressing the extracellular domain of LRP6 tagged with the immunoglobulin-γ Fc epitope (LRP6-IgG) or the extracellular domain of LDL receptor tagged with the immunoglobulin-γ Fc epitope (LDLR-IgG). Wnt3A bound to LRP6N-IgG, but not to LDLR-IgG (Fig. 10A), consistent with a previous report (Tamai et al., 2000). Notably, the Wnt3A palmitoylation mutant (C77S) bound less well to LRP6-IgG. In addition, we examined the ability of mFz8CRD-IgG to interact with Wnt3A and Wnt 3A C77S, and found that Wnt3A C77S also bound less well to mouse Fz8CRD (Fig. 10B).

Taken together, our results confirm the binding between Wnt and LRP and further demonstrate that palmitoylation of Wnt is important for its binding to both Frizzled and LRP.

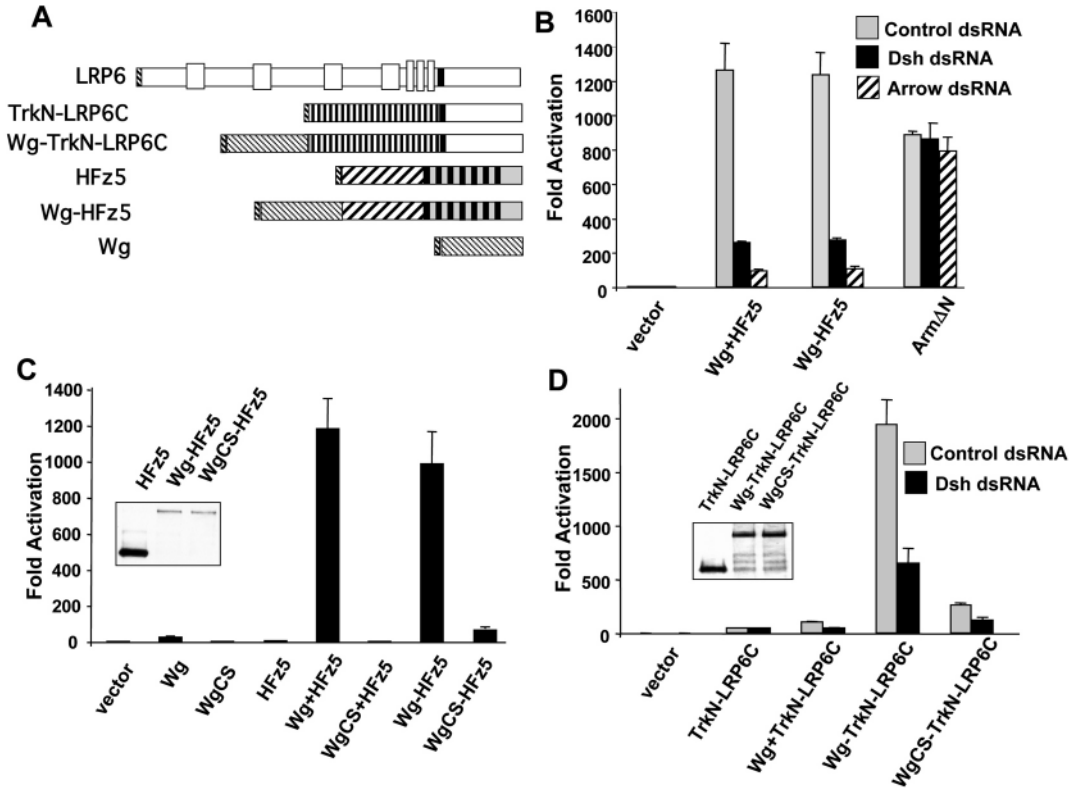

Fig. 9. Requirement of Wg can be by-passed by fusing Wg to human FZ5 or LRP6 and mutating the palmitoylation site of Wg reduces the activity of both chimeric receptors. (A) Schematic representation of human FZ5, TrkN-LRP6C, Wg-HFz5, Wg-TrkN-LRP6C. Wg was fused to the N terminus of human FZ5 to form Wg-HFz5. Both human FZ5 and Wg-HFz5 were fused with GFP at their C termini. Wg was also fused to the N terminus of TrkN-LRP6C to form Wg-TrkN-LRP6C. (B) Hyperactivity of Wg-HFz5 requires both Arrow and Dsh. S2 cells were treated with control-, Arrow- or Dsh-dsRNA, and transfected with the indicated plasmids. Luciferase activities were measured 48 hours after transfection. (C) Mutating C93, the potential palmitoylation site of Wg, reduces the signaling activity of Wg-HFz5. The membrane expressions of human FZ5, Wg-HFz5 and WgC93S-HFz5 were determined by membrane biotinylation and immunoblotting with anti-GFP antibodies (insert). (D) Wg-TrkN-LRP6C is a Dsh-dependent hyperactive chimeric receptor and mutating the palmitoylation site of Wg reduces the activity of this chimera. The membrane expressions of TrkN-LRP6C, Wg-TrkN-LRP6C and WgC93S-TrkN-LRP6C were determined by membrane biotinylation and immunoblotting with anti-TrkC antibodies (insert).

Discussion

Although the β-catenin degradation complex has been carefully studied (Peifer and Polakis, 2000), little is known about how a Wnt signal is transduced across the plasma membrane to regulate the β-catenin degradation complex. In this study, we have provided strong evidence supporting conclusions that the role of the extracellular domain of LRP is to interact functionally with Wnt proteins and that Wnt proteins act by forming hetero-oligomers of Frizzled and LRP.

What is the mechanism by which Frizzled transduces a Wnt signal? We have found that the mutations that disrupt the signaling activity of Frizzled also affect the ability of Frizzled to induce membrane translocation of Dvl and reduce physical interaction between Frizzled and Dvl (Fig. 3), suggesting that a physical interaction between Frizzled and Dvl is required for the signaling activity of Frizzled. We propose that Frizzled might function as a docking site for Dvl in β-catenin signaling. Our results are consistent with a previous finding that the Lys-Thr-x-x-x-Trp motif at the C-terminal tail of Frizzled is not only required for activating β-catenin signaling, but also for inducing Dvl membrane translocation (Umbhauer et al., 2000). Interestingly, a recent study has demonstrated that the PDZ domain of Dvl directly binds to a peptide of C-terminal region of Frizzled containing the Lys-Thr-x-x-x-Trp motif, and this peptide can inhibit Wnt/β-catenin signaling in *Xenopus* (Wong et al., 2003). However, the binding is relatively weak (K_d~10 μM). Our results suggest that multiple regions of Frizzled might be involved in the binding with Dvl and could increase the binding affinity.

The same structural elements may be required for Frizzled to function in both the planar polarity and the β-catenin pathways, as membrane translocation of Dvl has been implicated in planar polarity signaling (Axelrod, 2001; Rothbacher et al., 2000), and residues essential for the activity of Frizzled in β-catenin signaling are also important for Frizzled-induced translocation of Dvl to the plasma membrane (Fig. 3). It is possible that other proteins in the Frizzled-Dvl complex, such as LRP in β-catenin signaling and Flamingo in planar polarity signaling, determine the signaling consequences of interaction between Frizzled and Dvl.

What is the role of LRP in transmitting the Wnt signal and what is the function of its extracellular domain of LRP for receiving the Wnt signal? An in vitro binding assay has suggested that Wnt1 is able to bind to the extracellular domain

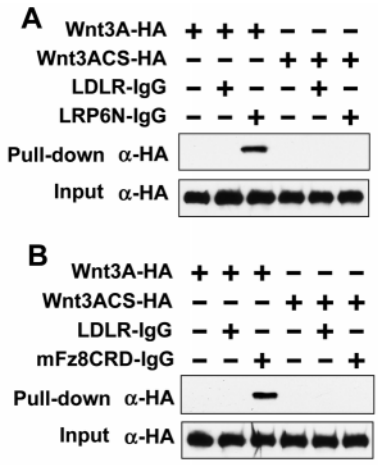

Fig. 10. Physical interactions between Wnt3A and its palmitoylation site mutant with the extracellular domain of LRP6 and mouse Fz8. Conditioned medium of Rat2 cells expressing HA-tagged Wnt3A or Wnt3A C77S was mixed with conditioned medium of 293 cells expressing LDLR-IgG (A,B), LRP6N-IgG (A), mFz8CRD-IgG (B), and incubated protein G-agarose beads. Precipitates were washed and fractionated by SDS-PAGE, and immunoblotted with anti-HA antibodies (upper panel). Input of Wnt3A in this pull down assay was determined by immunoblotting with anti-HA antibodies (lower panel).

of LRP (Tamai et al., 2000), but analogous binding was not observed in studies with Wg protein (Wu and Nusse, 2002). Results from in vitro binding assays need to be treated cautiously, as the concentrations of ligands and receptors in these assays could be significantly higher than in physiological situations, and certain components normally involved in formation of the receptor complex could be missing in these assays. Therefore, functional data are necessary to address the significance of potential binding between Wnt and LRP. We have shown that the extracellular domain of LRP can be functionally replaced by the extracellular domain of Frizzled (Fig. 6), suggesting a physiological role for a direct, or indirect, interaction of Wnt with the extracellular domain of LRP.

LRP can also transmit a signal via β-catenin without a requirement for Wnt (Mao et al., 2001a; Mao et al., 2001b; Schweizer and Varmus, 2003). We have taken advantage of two commonly used inducible oligomerization strategies to demonstrate that oligomerization of LRP6 increases its signaling activity and its interaction with Axin (Fig. 4). Interestingly, it has been shown that the second cysteine-rich domain of DKK2 stimulates β-catenin signaling via LRP independently of Dvl (Li et al., 2002). Further experiments are needed to determine whether this DKK2 fragment activates LRP by altering the oligomerization status of LRP.

A recent study has suggested that the extracellular domain of LRP might negatively regulate the signaling activity of LRP through dimerization, which can be relieved by Wnt proteins (Liu et al., 2003). By contrast, we have shown that the signaling activity of LRP was markedly increased by oligomerization (Fig. 4). The source of this discrepancy is currently unclear. However, we have found that, when overexpressed in 293 cells, full-length LRP6 was less efficiently transferred to the plasma membrane than was LRP6ΔN (Fig. 5), an observation that correlated with the lower signaling activity of LRP6. In addition, upon co-expression of MESD, a chaperone of LRP, the signaling activities of LRP6 and LRP6ΔN became equivalent (Fig. 5). These data suggest that the low signaling activity of full-length LRP6 is most likely due to its poor membrane localization, and strongly argue against a negative role of the extracellular domain of LRP in Wnt signaling. It should be also noted that only dimers of LRP6 could be induced in the previous study (Liu et al., 2003), whereas we have used LRP6 fusion proteins able to form oligomers. We have found that AP20187 increased the signaling activity of LRP6ΔEGF fused to two copies of FKBP (Fig. 4), but did not affect the signaling activity of LRP6ΔEGF fused to one copy of FKBP (data not shown). It is possible that in different conditions of dimer and oligomer formation, the intracellular domains of LRP6 are placed at different positions relative to each other, affecting the ability to bind to Axin.

Although Wnt can bind to both Frizzled and LRP, both receptors are essential for transducing the Wnt signal. It is possible that Wnt, Frizzled and LRP form one signaling complex. Alternatively, Wnt proteins might form separate complexes with Frizzled and LRP, which turn on separate signaling pathways that converge downstream.

We have provided several lines of evidence that support the first model, and our data suggest that bringing Frizzled and LRP into proximity is sufficient to trigger signaling through β-catenin signaling. We have shown that the Wnt signaling pathway can be fully stimulated by oligomerizing Frizzled and LRP either through the intracellular region, by directly

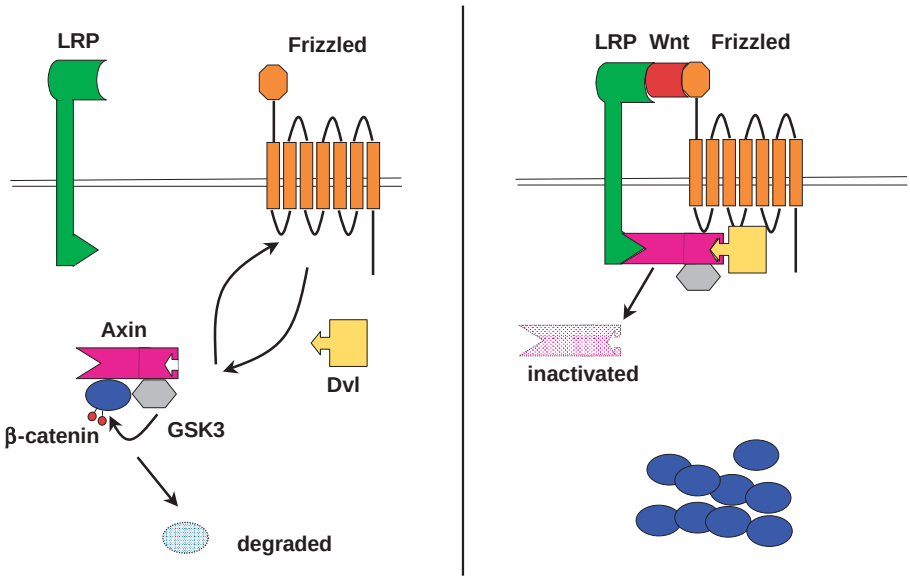

Fig. 11. A model for transducing the Wnt signal by Frizzled and LRP (see Discussion).

fusing the intracellular domain of LRP6 to the C terminus of human FZ5 (Fig. 7), or through the extracellular region, by co-expressing TrkN-LRP6C and NT3-HFz5 (Fig. 8). Furthermore, we have shown that the requirement for free Wnt proteins can be bypassed by fusing Wnt to either Frizzled or LRP (Fig. 9). These results suggest that Wnt, Frizzled and LRP form a single signaling complex, and the function of Wnt is to form a bridge between Frizzled and LRP. We recognize, however, that Wnt-induced oligomerization of endogenous Frizzled and LRP in living cells has not been demonstrated, nor have we characterized the physical properties of the proposed Wnt-induced oligomers.

Why is it necessary and sufficient to bring LRP and Frizzled into proximity for transducing the Wnt signal? Our RNA interference studies have indicated that signaling by overexpressed LRP is strictly Dvl independent, and Dvl becomes important once Wnt and Frizzled are involved. Axin is known to interact with the C terminus of LRP (Mao et al., 2001b; Tolwinski et al., 2003), and Dvl can interact with Frizzled (Fig. 3) (Wong et al., 2003). Presumably, once overexpressed, a high concentration of membrane LRP is able to bring endogenous Axin to the plasma membrane, based solely on its affinity with Axin, so that Axin might be inactivated or degraded (Mao et al., 2001b; Tolwinski et al., 2003; Willert et al., 1999). This would explain why the signaling activity of ectopically expressed LRP is Dvl independent. Under normal physiological conditions, Frizzled and Dvl might be required to translocate Axin to the membrane LRP upon Wnt signaling. Dvl might function as a molecular chaperone to deliver Axin to the Frizzled-LRP complex, based on its affinity with both Frizzled and Axin. In addition, Frizzled and Dvl might also enhance the binding affinity between LRP and Axin through promoting phosphorylation of LRP (Tamai et al., 2004). Therefore, the Wnt-Frizzled-LRP complex might serve as a high-affinity docking site for Axin (Fig. 11). This model is also in agreement with the recent finding that Wnt induces translocation of Axin to the membrane in a Dvl-dependent manner (Cliffe et al., 2003). Consistent with its proposed role as a shuttle, Dvl is associated with intracellular vesicles, and interacts with both actin stress fibers and microtubules (Capelluto et al., 2002; Miller et al., 1999b).

In the model illustrated in Fig. 11, Wnt stimulates the β-catenin pathway by relocating Axin to the plasma membrane and inactivating Axin. It is still not clear whether Wnt-induced Axin membrane translocation is a prerequisite for dissociation of the β-catenin degradation complex. Furthermore, it is unknown whether the only function of Dvl is to facilitate the transport of Axin to the plasma membrane. It is possible that Dvl also brings certain factors to Axin upon Wnt signaling and promotes inactivation of the Axin complex. Indeed, it has been suggested that in response to Wnt, Dvl can recruit Frat/GBP, a strong inhibitor of GSK3 (Yost et al., 1998), to the Axin-GSK3-β-catenin complex (Li et al., 1999), although a requirement for Frat/GBP in Wnt signaling has not been established genetically. Furthermore, it is unclear whether inhibition of GSK3 normally plays a major role in Wnt signaling, although dominant-negative mutants of GSK3 can activate β-catenin signaling (He et al., 1995). It has been shown that Wnt induces dephosphorylation of Axin (Willert et al., 1999; Yamamoto et al., 1999), which might reflect inhibition of GSK3 or dissociation of the Axin-GSK3 complex. Dephosphorylated Axin appears to be less stable (Yamamoto et al., 1999) and binds β-catenin less efficiently (Willert et al., 1999). It is currently unknown how membrane translocation of Axin is coupled to dephosphorylation and destabilization of Axin. More work will be necessary to illustrate fully the molecular mechanism by which Wnt induces the stabilization of β-catenin.

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