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Role of the Intracellular Domains of LRP5 and LRP6 in Activating the Wnt Canonical Pathway

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Abstract LDL-receptor related proteins 5 and 6 (LRP5/6) are co-receptors of Frizzled receptors that mediate Wnt-induced activation of the transcription factor family TCF/LEF-1. Even though LRP5 and LRP6 are highly homologous, LRP6, but not LRP5, is expressed primarily in the nervous system and deletion of the LRP6 gene results in significant brain abnormalities, while deletion of LRP5 results in primarily decreased bone density. Additionally, the exact function of LRP5 and LRP6 have not been clearly defined, although it is clear that they both play key roles in the Wnt canonical pathway. In this study the role of the intracellular domains of LRP5/6 in mediating Wnt signaling was examined. In the absence of exogenous Wnt 3a, full-length (FL) LRP6, but not LRP5, increased TCF/LEF-1 transcriptional activity, however both significantly potentiated Wnt 3a-induced TCF/LEF-1 activation. In contrast to the findings with the FL constructs, the intracellular domains (membrane-anchored and cytosolic) of both LRP5 and LRP6 significantly increased TCF/LEF-1 activation in the absence of Wnt 3a, and potentiated the Wnt 3a-induced decrease in β-catenin phosphorylation, increase in free β-catenin levels and the increase in TCF/LEF-1 activity. These findings demonstrate that: (1) LRP5 and LRP6 differentially modulate TCF/LEF-1 activation in the absence of Wnt 3a and (2) the intracellular C-terminal domains of LRP5/6 potentiate Wnt 3a-induced TCF/LEF-1 activation whether or not they are membrane-anchored. These findings provide significant new insights into the roles of LRP5/6 in modulating canonical Wnt signaling. J. Cell. Biochem. 95: 328–338, 2005. © 2005 Wiley-Liss, Inc.

Key words: Wnt signaling; β-catenin; TCF/LEF-1; LRP5/6

Wnts are secreted glycoproteins that play an essential role in embryonic induction, patterning of the body axis, and regulating proliferation and cell fate determination in the dorsal neural tube and neural crest (for a review see [Wodarz and Nusse, 1998]). Wnts are also required for normal development of the hippocampus [Lee et al., 2000] and the cortex [Grove et al., 1998]. In the adult vertebrate, Wnt signaling also plays important roles in regulating such processes as neuronal plasticity [Dale, 1998; Hall et al., 2000] and tissue homeostasis [Wodarz and Nusse, 1998; Seidensticker and Behrens, 2000; Miller, 2002]. Given the diverse effects elicited by Wnts, it is not surprising that there are a large number of Wnts; 19 have been identified in the human genome [He, 2003]. The most well-established mechanism by which Wnts elicit their biological effects is by interaction with members of the Frizzled (Fz) family of receptors [Yang-Snyder et al., 1996; He et al., 1997; Slusarski et al., 1997; Holmen et al., 2002; Karasawa et al., 2002]. Fz receptors all have seven transmembrane domains and ten different human Fz receptors have been identified [Huelsken and Birchmeier, 2001]. Wnt binding to specific Fz receptors has been shown to result in activation of c-Jun N-terminal kinase, protein kinase C or β-catenin signaling cascades [Kuhl et al., 2000; Seidensticker and Behrens, 2000; Huelsken and Birchmeier, 2001; Pandur et al., 2002]. The type of Wnt, Fz receptor, and also the presence of other interacting and/or regulatory proteins determine which signaling pathway is activated [Boutros et al., 2000; Rulifson et al., 2000; Wu and Nusse, 2002].

The best characterized of the three Wnt signaling cascades is the β-catenin or canonical pathway [Seidensticker and Behrens, 2000].

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Wnt canonical signaling is transduced through β-catenin which is regulated by the adenomatous polyposis coli protein (APC)/axin/glycogen synthase kinase 3β (GSK3β) complex. In the absence of Wnt signal, β-catenin is phosphorylated by casein kinase 1 and subsequently by GSK3β in this complex [Liu et al., 2002]. The phosphorylated β-catenin is ubiquitinated and degraded by the proteasome pathway. When specific Wnts (e.g., Wnt 3a) bind to Fz receptors this results in the activation of disheveled (Dsh) and other proteins, which facilitates the dissociation of the β-catenin-APC/axin/GSK3β complex and prevents β-catenin from being efficiently phosphorylated. The unphosphorylated cytosolic β-catenin is not ubiquitinated and targeted to the proteasome, leading to increased cytosolic β-catenin levels. β-catenin subsequently translocates to the nucleus, where it binds to members of the T-cell factor (TCF)/ lymphocyte enhancer factor (LEF) 1 transcription factor family and turns on expression of target genes [Dale, 1998; Gumbiner, 1998; Wodarz and Nusse, 1998].

In 2000, another important component of the Wnt canonical pathway was identified. It was demonstrated that *Drosophila* Arrow or the mammalian low-density lipoprotein (LDL)-receptor related proteins 5 and 6 (LRP5 and 6) were co-receptors for the Wnt canonical signaling pathway [Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000]. LRP5/6, as well as Arrow, are single pass transmembrane receptors. They are in the LDL-receptor family, generally recognized as cell surface endocytic receptors, which bind and internalize extracellular ligands for degradation by lysosomes [Li et al., 2001]. LRP5 and LRP6 are highly homologous (their protein sequences are 73% identical), but the ligand-binding repeats show only 50% similarity, which means they may bind related, but not identical ligands. Further, it has been demonstrated that expression of several Wnt-Fz fusion proteins with LRP6, but not LRP5, resulted in TCF/LEF-1 activation [Holmen et al., 2002] and LRP6, but not LRP5, overexpression resulted in axis duplication in *Xenopus* [Tamai et al., 2000]. These findings indicate that LRP6 may be more active than LRP5 in the Wnt canonical pathway.

The expression patterns of LRP5 and LRP6 differ. Human LRP5 gene is highly expressed in many tissues and involved in bone development [Gong et al., 2001], cholesterol metabolism, and the modulation of glucose-induced insulin secretion [Hey et al., 1998; Kim et al., 1998; Figueroa et al., 2000]. LRP5 knockout mice are viable and fertile, although they do exhibit low bone mass and eye vascularization defects [Kato et al., 2002]. In contrast, the human LRP6 gene is expressed primarily in the nervous system, and disruption of the mouse LRP6 gene results in death at birth and developmental defects that are very similar to those that result from mutations in Wnt genes, specifically Wnt 3a, Wnt 1 and Wnt 7a [Pinson et al., 2000]. These defects include neural tube closure defects, limb defects and mid/hindbrain defects [Pinson et al., 2000]. Further analysis of the LRP6 null mice revealed that there was reduced production of dentate granule neurons and abnormalities in radial glial scaffolding without defects in the pyramidal cell fields [Zhou et al., 2004]. These studies demonstrate the importance of LRP6 in the development of the CNS.

Although it is clear that LRP5/6 play an important role in the Wnt-induced activation of the β-catenin canonical pathway, the mechanisms involved have not been fully elucidated. There is data to suggest that axin interacts with the intracellular domain of LRP5 in vitro and in vivo, and the addition of Wnt-1 appears to stimulate the recruitment of axin to LRP5 at the membrane, where axin is degraded, which subsequently results in increased β-catenin levels and signaling [Mao et al., 2001; Tolwinski et al., 2003]. Recent data has also provided evidence that the PPPSP motif in the intracellular domain of LRP5/6 is required for signaling [Brennan et al., 2004] and that phosphorylation of this motif creates a docking site for axin [Tamai et al., 2004]. There is also data to suggest that Wnt may signal through LRP5/6 directly to the β-catenin destruction complex in a Fz receptor- and Dsh-independent fashion [Mao et al., 2001; Li et al., 2002]. However overall, much remains unknown as to how LRP5/6 are activated by Wnt signaling and whether forming a complex between LRP5/6, Wnt and Fz is required for transducing an efficient signal to stabilize β-catenin. It is also unclear whether LRP5 and LRP6 play the same roles in the cell [Holmen et al., 2002].

In the present study, we clearly demonstrate that in the absence of exogenously added Wnt 3a, FL LRP6, but not LRP5, activates TCF/LEF-1 in a mammalian cell system. Moreover, we show that the intracellular domains of both

LRP5 and LRP6 not only constitutively increase free β-catenin levels and activate TCF/LEF-1 as has been previously demonstrated [Mao et al., 2001], but they also potentiate Wnt 3a signaling, whether or not they are membrane anchored. Taken as a whole, these findings indicate that recruitment of axin to the membrane is not the only mechanism by which LRP5/6 activate the Wnt canonical signaling pathway, and it is therefore likely that LRP5/6 may facilitate β-catenin signaling through multiple mechanisms.

MATERIALS AND METHODS

Preparation of LRP5/6 Constructs

PCR amplification was carried out using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA). All constructs were subcloned into the pCMV 5A vector (Invitrogen, Carlsbad, CA), which results in a Myc-tag at the C-terminal (Fig. 1A). To make the LRP5 constructs, mouse LRP5 cDNA (Orbigen, San Diego, CA) was used as the template. Primers sequences were as follows for PCR: forward 5′-GTA AGA GTG CGG CCG CTA TAA TGG AAA CGG CGC CGA CCC-3′, reverse 5′-CCT AAG CTT GGA CGA GTC CGT GCA GGG GGA CGG TGG G-3′ for LRP5; forward 5′-CTA AGA AGT GCG GCC GCT ATG GGT GGA GCC CCT CAT GTG CCT CTC AAC-3′, reverse 5′-CCT AAG CTT GGA CGA GTC CGT GCA GGG GGA CGG TGG G-3′ for LRP5 cytosolic domain only (LRP5-C3). The PCR reaction product was digested with *Not I* and *Hind III* and ligated into the *Not I* and *Hind III* sites of pCMV 5A. For LRP5-C2, in which the extracellular domain of the FL-LRP5 was deleted but the N-terminal signal peptide, transmembrane and cytosolic domains were maintained, FL-LRP5 in pCMV 5A was used as the template and the fragment was amplified by using the primers: forward 5′-GCT CTA GAG ACT CCT TCC CCG ACT GTG CTG ATG GGT CTG ATG AG-3′, and reverse 5′-GCT CTA GAG GCC GCG GCG GGG ACC AAG-3′. The resulting PCR product was digested with *Xba I* and ligated back on itself, which resulted in an LRP5 construct with only the extracellular domain deleted. To make the LRP6 constructs, human LRP6 in pCS2+ was used as the template, which was a generous gift from Dr. Xi He (Harvard Medical School, Boston, MA). The following primers were used for each PCR reaction: forward 5′-CGC GGA TCC AAT ATG GGG GCC GTC CTG AGG AGC CTC CTG-3′, reverse 5′-GCG CGT CGA CGG AGG AGT CTG TAC AGG GAG AGG GTG GCG GCG GTG GGT-3′ for FL-LRP6; forward 5′-CGC GGA TCC AGC ATG GGA CCA GCT TCT GTG CCT CTT GGT TAT GTG-3′, reverse 5′- GCG CGT CGA CGG AGG AGT CTG TAC AGG GAG AGG GTG GCG GCG GTG GGT-3′ for LRP6-C3. The PCR reaction product was digested with *BamH I* and *Sal I* and ligated into the *BamH I* and *Sal I* sites of pCMV 5A. The LRP6-C2 was made like the LRP5-C2 construct, using FL-LRP6 in pCMV 5A as the template and the primers: forward 5′- GGC TCT AGA CAT AAT GTG GAT TGC AGT GAC AAG TCA GAT GAA CTG GAT T-3′ and reverse 5′- GCG TCT AGA GGC CGC TCT CAG GAG CAC ACA GAA-3′. The resulting PCR product was digested with *Xba I* and ligated back on itself. The integrity of all constructs was confirmed by sequence analysis.

Cell Culture

Chinese hamster ovary (CHO) cells were grown in Ham's F-12/Dulbecco's modified Eagle medium (DMEM; Irvine Scientific, Santa Ana, CA) supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT), 100 U/ml penicillin (Gibco, Carlsbad, CA), 100 μg/ml streptomycin (Gibco), and 2 mM L-glutamine (Gibco). COS-7 (ATCC, Manassas, VA) cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Both cell lines were maintained in a humidified 5% CO_2 incubator at 37°C.

Preparation of Conditioned Media

L-cells stably transfected with Wnt 3a (L-Wnt 3a cells) (ATCC, Manassas, VA) were cultured in DMEM containing 10% FBS and 400 μg/ml G418 (Alexis Biochemicals, San Diego, CA). Wnt 3a-conditioned medium (Wnt 3aCM) was prepared following the protocol provided by ATCC. The control-conditioned medium (LCM) was prepared from the parental cell line, L-M (TK-) cells (ATCC), using the same protocol as was used for the stably transfected L-Wnt 3a cells.

Immunoblot Analysis

To analyze the expression of the constructs, CHO cells were transiently transfected with the indicated constructs and 48 h later were rinsed in ice-cold phosphate-buffered saline (PBS) and collected in cold lysis buffer containing 2%

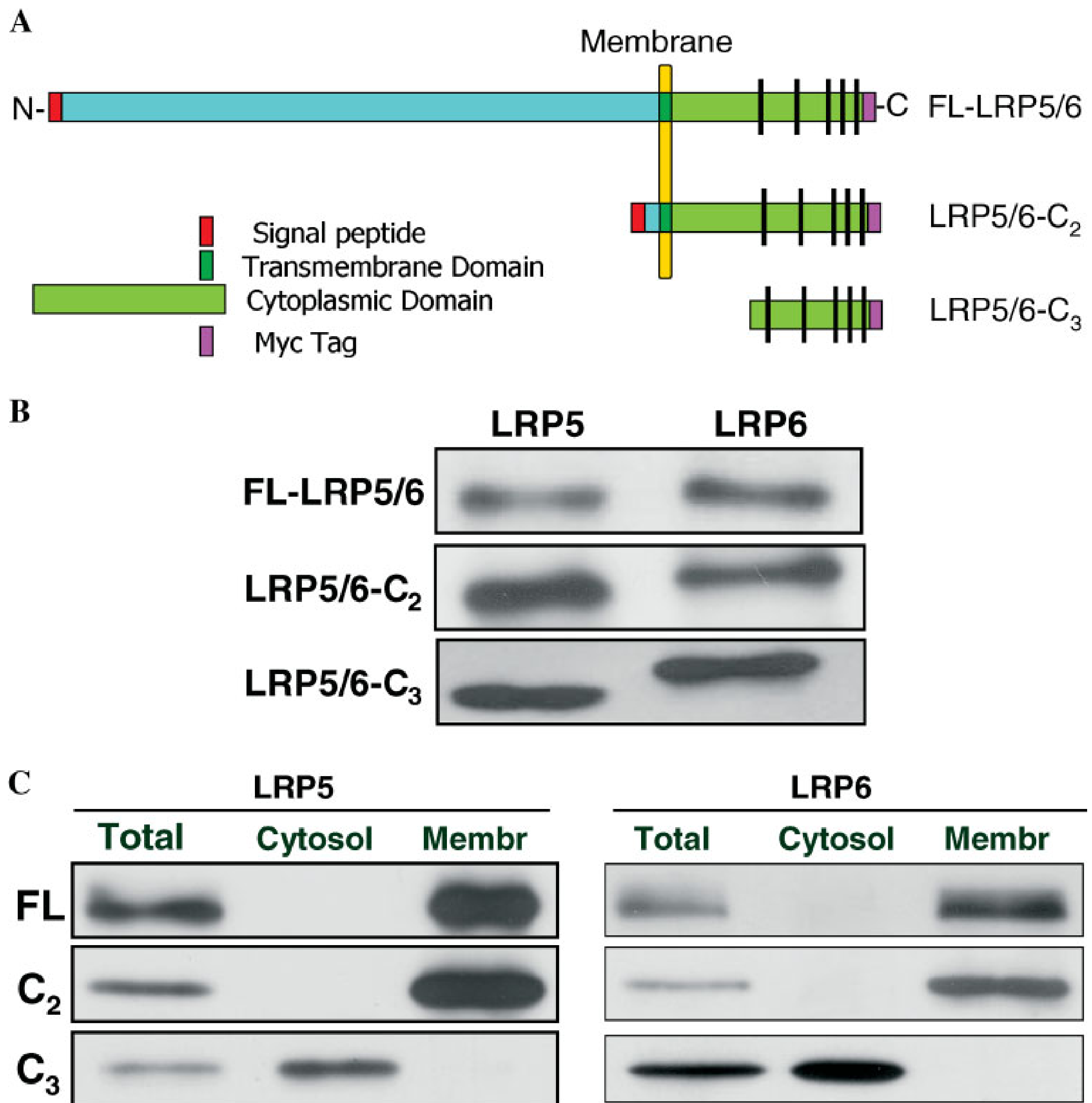

Fig. 1. LRP5/6 constructs and their expression. **A**: Schematic representation of the LRP5/6 constructs and their structural features. **B**: CHO cells were transiently transfected with FL-LRP5/6, LRP5/6-C2, or LRP5/6-C3 and 48 h later the cells were collected. Lysates (20 μg) were immunoblotted using a Myc antibody. The blots show that the expression levels of the LRP5 and LRP6 constructs are similar. **C**: Subcellular localization of LRP5/6 constructs. Cells were transfected with the indicated LRP5/6 constructs, cytosolic and membrane fractions were prepared and immunoblotted using a Myc antibody. These results demonstrate that as expected, FL-LRP-5/6, and LRP5/6-C2 exclusively localize to the membrane (Membr) fraction. In contrast, LRP5/6-C3 was found only in the cytosolic fraction.

sodium dodecyl sulfate (SDS), 250 mM Tris-Cl (pH 7.4), 10% glycerol, 5 mM EDTA, and 5 mM EGTA, 0.1 mM PMSF, 0.1 μM okadaic acid, and 10 μg/ml concentration each of aprotinin, leupeptin, and pepstatin; sonicated on ice, boiled 10 min and spun at 16,000*g* at 4°C for 10 min. Protein concentrations of the supernatant were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Samples were diluted to 1 μg/μl in 2× SDS stop buffer [0.25M Tris-HCl, pH 7.5, 2% SDS, 25 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, 10% glycerol, and 0.01% bromophenol blue], and incubated in a boiling water bath for 10 min. Samples were

resolved on 5%–12% gradient SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. Membranes were then rinsed with TBST and incubated overnight at 4°C with a mouse monoclonal Myc antibody prepared by the University of Alabama at Birmingham Hybridoma Core facility. Blots were then rinsed with TBST and incubated with peroxidase-conjugated rabbit anti-mouse antibody in 5% milk in TBST for 1 h at room temperature. The blots were then rinsed with TBST several times and developed using enhanced chemiluminescence (ECL) (Amersham Pharmacia, Arlington Heights, IL).

For determination of free phospho-β-catenin levels, COS-7 cells were transiently transfected for 24 h and subsequently incubated in LCM or Wnt 3aCM for 2 h. Cells were then rinsed with ice-cold PBS, collected in a detergent-free hypoosmotic lysis solution (10 mM Tris-Cl, pH 7.4, 1 mM EGTA, and 1 mM EDTA, with protease inhibitors), incubated on ice for 15 min and sonicated for 1 s. A small aliquot of the total lysate was collected prior to centrifuging the samples at 100,000*g*. Supernatants were collected, and protein concentrations were determined using the BCA assay and diluted in 2× SDS buffer with dye and DTT and electrophoresed on 8% SDS polyacrylamide gels and transferred to nitrocellulose. Blots were then probed with a phospho-β-catenin polyclonal antibody (phospho-Ser 33/37, Thr 41, Cell Signaling, Beverly, MA) and visualized as above.

For the determination of free β-catenin levels, a pGST-E-cadherin construct was made using RNA from HEK293 cells exactly as described previously except that the E-cadherin was subcloned into pGEX-6P (Amersham Pharmacia Biotech) [Bafico et al., 1998]. The pull down assay was carried exactly as previously described using pGST-E-cadherin and GST control proteins [Bafico et al., 1998], and the precipitates were probed with a β-catenin monoclonal antibody (BD Biosciences).

Preparation of Cytoplasmic and Membrane Fractions

Cells were washed twice in ice cold PBS and scraped gently into buffer containing 0.25M sucrose, 5 mM HEPES, pH 7.5, 1 mM EGTA, 1 mM EDTA with protease inhibitors. Cells were lysed by 15 strokes in a chilled Teflon/glass homogenizer on ice. The lysates were centrifuged at 3,500*g* for 5 min to remove unbroken cells and nuclei. The cleared lysates were subject to centrifugation at 100,000*g* for 30 min at 4°C. The supernatants were collected as the cytoplasmic fraction and the pellets were resuspended in lysis buffer as the membrane fraction. Protein concentrations were determined by using the BCA assay, and samples were diluted into 2× SDS stop buffer before incubating in a boiling water bath. Samples were immunoblotted as described above.

TCF/LEF-1 Activity Assay

COS-7 cells were grown in 12-well plates and transfected with the indicated constructs, 0.2 μg Topflash-luciferase reporter (Upstate, Charlottesville, VA), 0.2 μg LEF-1 (from Dr. R Grosschedl), and 0.005 μg of pRL-TK (Promega, Madison, WI), which encodes a *Renilla* luciferase gene downstream of a minimal HSV-TK promoter and was included in each transfection to control for transfection efficiency. The transfections were carried using Fugene 6 (Roche, Basel, Switzerland) and the final amount of cDNA transfected into the cells was always made equivalent by using a control β-galactosidase (LacZ) construct. In some experiments the cell media was changed to Wnt 3aCM or LCM 7 h after transfection. Forty-eight hours after transfection, the cells were lysed in lysis buffer (Promega, Madison, WI) by shaking at room temperature for 20 min. The luciferase activity was then measured using the Promega Dual-Luciferase reporter assay system with a luminometer (Turner Designs, Sunnyvale, CA). All experiments were performed three times, and each time the measurements were done in triplicate.

Statistics

Data were analyzed using ANOVA, and values were considered significantly different when $P < 0.05$. Results were expressed as mean ± SE.

RESULTS

LRP5/6 Constructs and Their Localization

To analyze the roles of LRP5 and LRP6 in modulating the canonical Wnt signaling pathway in mammalian cells, FL LRP5 and LRP6 were subcloned into the pCMV 5A vector, which

results in C-terminal Myc-tagged constructs (FL-LRP5/6). LRP5 and LRP6 constructs lacking the extracellular (LRP5/6-C2) domains were also generated, as well as constructs of just the intracellular domain and hence not anchored at the membrane (LRP5/6-C3) (Fig. 1A). CHO cells were transiently transfected with FL-LRP5/6, LRP5/6-C2, or LRP5/6-C3. Lysates were collected and immunoblotted with the Myc antibody. The expression levels of the LRP5 and LPR6 constructs were similar (Fig. 1B). To analyze the intracellular distribution of the constructs, cell were transfected with the indicated LRP5 or LRP6 constructs, and cytosolic and membrane fractions were prepared and immunoblotted using the Myc antibody. The results show, as expected, that FL-LRP5/6 and LRP5/6-C2 exclusively localize to the membrane fraction. In contrast, LRP5/6-C3 was only in the cytosolic fraction (Fig. 1C).

FL-LRP5 and FL-LRP6 Differentially Affect TCF/LEF-1 Activation in the Absence of Wnt 3a, But Facilitate TCF/LEF-1 Activity Similarly in Response to Wnt 3a

In the absence of an activated Wnt signaling cascade, phosphorylated β-catenin is targeted for ubiquitination and degradation by the proteasome preventing accumulation and nuclear translocation. To determine whether FL-LRP5 or FL-LRP6 can activate the Wnt canonical signaling pathway in the absence of exogenously added Wnt, cells were transfected with FL-LRP5 or FL-LRP6 and TCF/LEF-1 activation was measured. LacZ transfected cells were used as controls. FL-LRP5 alone in the absence of Wnt did not activate the Topflash reporter. In contrast, TCF/LEF-1 activity was significantly increased in cells transfected with 0.5 μg and 1 μg FL-LRP6 (Fig. 2A). This result indicates that FL-LRP6, but not FL-LRP-5, can activate the Wnt signaling cascade in the absence of exogenously added Wnt. However both FL-LRP5 and FL-LRP6 potentiate Wnt 3a-mediated TCF/LEF-1 activation (Fig. 2B).

Removal of the Extracellular Domain of LRP5 and LRP6 Results in Constitutive Activation and Potentiation of Wnt 3a-Induced TCF/LEF-1 Activity

To evaluate the role of the intracellular and extracellular domains of LRP5 and LPR6 in modulating the Wnt signaling cascade, we prepared several deletion constructs of LRP5 and LRP6. LRP5-C2 includes the entire intracellular domain, the transmembrane domain and a very short stretch of the extracellular domain starting at residue 1356. LRP6-C2 is identical to LRP5-C2 except the highly truncated extracellular domain starts at residue 1346, which is the same site as the LRP5-C2 construct based on alignment of mouse LRP5 and human LRP6 amino acid sequences. In the absence of Wnt 3a, LRP5-C2, and LRP6-C2 significantly increased TCF/LEF-1 activity, which was comparable to Wnt 3a-induced TCF/LEF-1 activity (Fig. 3A). These results indicate that removing the extracellular domain of both LRP5 and LRP6 leads to

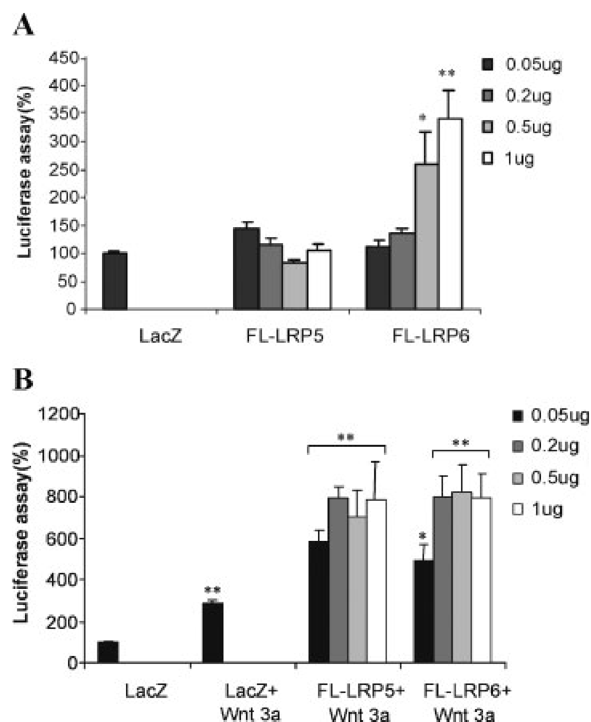

Fig. 2. FL-LRP5 and FL-LRP6 differentially effect TCF/LEF-1 activation in the absence of Wnt 3a, but facilitate TCF/LEF-1 activity similarly in response to Wnt 3a. **A**: COS-7 cells were transfected with Topflash luciferase reporter, LEF-1, *Renilla* luciferase and the indicated amounts of FL-LRP5, FL-LRP6, or LacZ. Forty-eight hours later luciferase activity was measured. The results demonstrate that FL-LRP5 alone did not activate TCF/LEF-1. However, FL-LRP6 significantly increased TCF/LEF-1 activity at 0.5 μg and 1 μg compared with control cells (LacZ) (* $P < 0.05$, ** $P < 0.01$). **B**: After COS-7 cells were transfected as in (A) for 7 h, cells were treated with Wnt 3a CM for another 42 h. The LacZ cells (control) were treated with LCM. Wnt 3a significantly increased TCF/LEF-1 activity compared to control LacZ cells (** $P < 0.01$). Both FL-LRP5 and FL-LRP6 strongly potentiated Wnt 3a-induced TCF/LEF-1 activity compared with Wnt 3a treatment alone (* $P < 0.05$, ** $P < 0.01$).

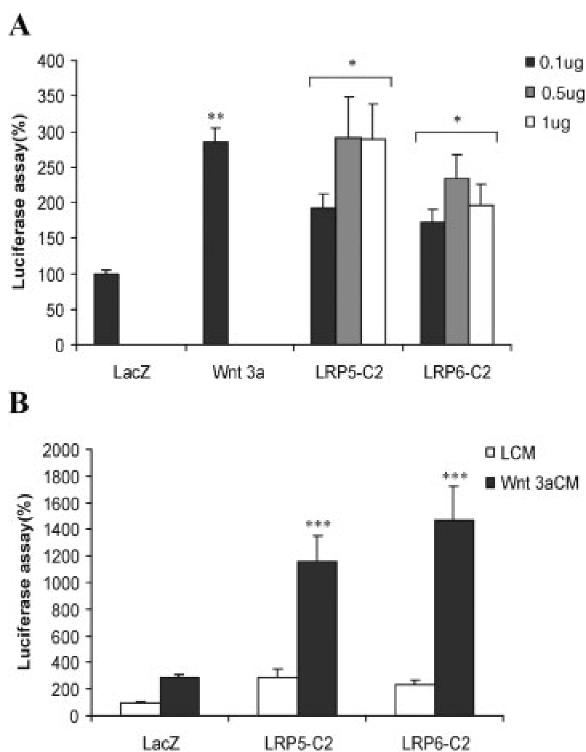

Fig. 3. Removal of the extracellular domain of LRP5 and LRP6 results in constitutive activation, and potentiation of Wnt 3a-induced TCF/LEF-1 activity. **A**: TCF/LEF-1 activity was measured in COS-7 cells transfected with 0.1–1 μg LRP5-C2 or LRP6-C2 with the Topflash luciferase reporter, LEF-1 and *Renilla* luciferase for 48 h. Compared to the control LacZ cells, both LRP5-C2 and LRP6-C2 significantly increased TCF/LEF-1 activity, which was comparable to Wnt 3a-induced TCF/LEF-1 activity (*$P < 0.05$, **$P < 0.01$). **B**: Both LRP5-C2 and LRP6-C2 potentiate Wnt 3a-induced TCF/LEF-1 activity. COS-7 cells were transfected with 0.5 μg LRP5-C2 or LRP6-C2 with the Topflash luciferase reporter, LEF-1 and *Renilla* luciferase for 7 h, followed by incubation in Wnt 3aCM or LCM as indicated for 42 h. Wnt 3a-induced TCF/LEF-1 activity was significantly greater in cells transfected with LRP5-C2 or LRP6-C2 cells compared with Wnt 3a-treated LacZ-transfected cells (***$P < 0.001$).

constitutive activation of the intracellular domain. The constitutively active nature of the C2 mutants suggests that the intracellular domain of LRP5 and LRP6 is actively involved in transducing Wnt signals. Interestingly, Wnt 3a-induced TCF/LEF-1 activity was significantly greater in cells transfected with LRP5-C2 or LRP6-C2 cells compared with Wnt 3a-treated LacZ-transfected cells (Fig. 3B). This data demonstrated that LRP5 and LRP6 without the extracellular domain could still facilitate Wnt 3a-induced TCF/LEF-1 activation.

Cytosolic Domain of LRP5/6 Can Constitutively Activate TCF/LEF-1 and Potentiate the Wnt 3a-Induced TCF/LEF-1 Activity

A previous study had shown that LRP5-C2 constitutively activates TCF/LEF-1 in the absence of Wnt [Mao et al., 2001], and our findings are in agreement with these previous results. It was also proposed that the membrane anchored LRP5-C2 acts by recruiting axin to the membrane where it is degraded [Mao et al., 2001]. To examine the role of just the intracellular domains of LRP5 and LRP6, deletion constructs of just the cytosolic domain were prepared. LRP5-C3 includes only the intracellular domain, starting at residue 1428. LRP6-C3 is identical to LRP5-C3 except the intracellular domain starting at residue 1416. As shown in Figure 1C, these constructs localize in the cytosolic fraction. Surprisingly, we found that both LRP5-C3 and LRP6-C3 constitutively activate TCF/LEF-1-dependent transcription in COS-7 cells in the absence of exogenously added Wnt 3a (Fig. 4A) in a manner similar to the C2 constructs (Fig. 3A). Thus, the intracellular domains of LRP-5 and LRP-6 constitutively activate the Wnt canonical pathway independent of Wnt 3a whether or not they are membrane-anchored. Further both LRP5-C3 and LRP6-C3, like LRP5-C2 and LRP6-C2, significantly increased Wnt 3a-induced TCF/LEF-1 activity compared with Wnt 3a treated control LacZ cells (Fig. 4B). Expression of either LRP5-C2 or LRP5-C3 significantly decreased the levels of cytosolic phospho-β-catenin and robustly potentiated the Wnt 3a-induced decrease in cytosolic phospho-β-catenin levels (Fig. 5A). Further, both LRP5-C2 and LRP5-C3 increased free β-catenin levels to the same extent as treatment with Wnt 3a (Fig. 5B), and significantly potentiated the increase in the levels of free β-catenin induced by Wnt 3a treatment (Fig. 5C). Similar results were obtained with LRP6-C2 and LRP6-C3, and were comparable to LRP5-C2 (Fig. 5D). These results clearly demonstrate that the intracellular domains of LRP5 and LRP6 can potentiate the canonical Wnt signal in the absence of the extracellular domains.

DISCUSSION

The aim of this study was to gain insight to the role of LRP5 and LRP6 in modulating TCF/LEF-1 activation in absence or presence of Wnt 3a.

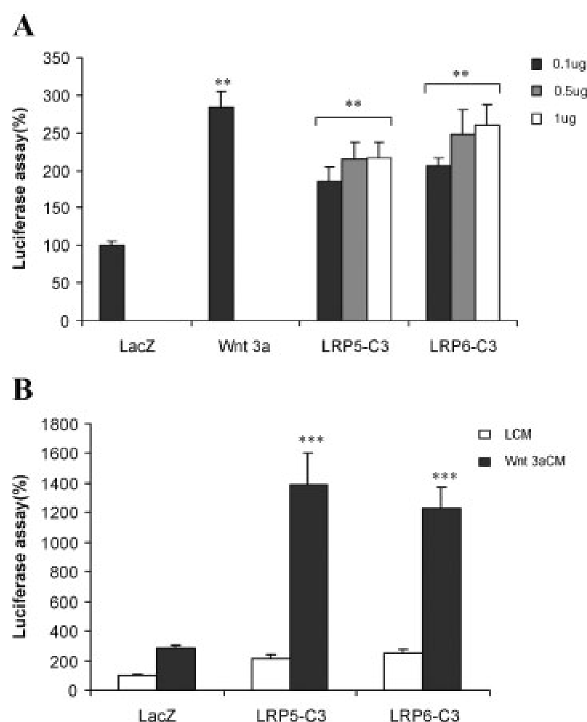

Fig. 4. The cytosolic domain of LRP5/6 can constitutively activate TCF/LEF-1 and potentiate the Wnt 3a-induced TCF/LEF-1 activity. **A**: The cytosolic domains of both LRP5 and LRP6 constitutively activate TCF/LEF-1 activity in COS-7 cells. Cells were transfected with 0.1–1 μg LRP5-C3 or LRP6-C3 with the Topflash luciferase reporter, LEF-1 and *Renilla* luciferase and 48 h later TCF/LEF-1 activities was measured. Both LRP5-C3 and LRP6-C3 significantly increased TCF/LEF-1 activity in the absence of Wnt 3a, and was comparable to the activity observed with Wnt 3a alone (**$P < 0.01$). **B**: Both LRP5-C3 and LRP6-C3 potentiate the Wnt 3a-induced TCF/LEF-1 activity. LRP5-C3 (0.5 μg) or LRP6-C3 cDNA with the reporter genes were transfected into COS-7 cells. Seven hours later the media was changed to Wnt 3aCM or LCM as indicated and cells were incubated for another 42 h. Both LRP5-C3 and LRP6-C3 significantly increased Wnt 3a-induced TCF/LEF-1 activity compared with Wnt 3a treated control LacZ cells (***$P < 0.001$).

The results presented in this study clearly demonstrate that in the absence of exogenously added Wnt 3a, only FL LRP6 constitutively activates TCF/LEF-1. Furthermore, we also demonstrate that the intracellular domains of both LRP5 and LRP6 not only constitutively activate TCF/LEF-1, but they also potentiate of Wnt 3a signaling whether or not they are membrane-anchored. These finding provide important insights into the role LRP5 and LRP6 play in modulating Wnt function and TCF/LEF-1 activation.

Wnt signaling through β-catenin has been studied extensively, however much remains unknown about this pathway, mostly with

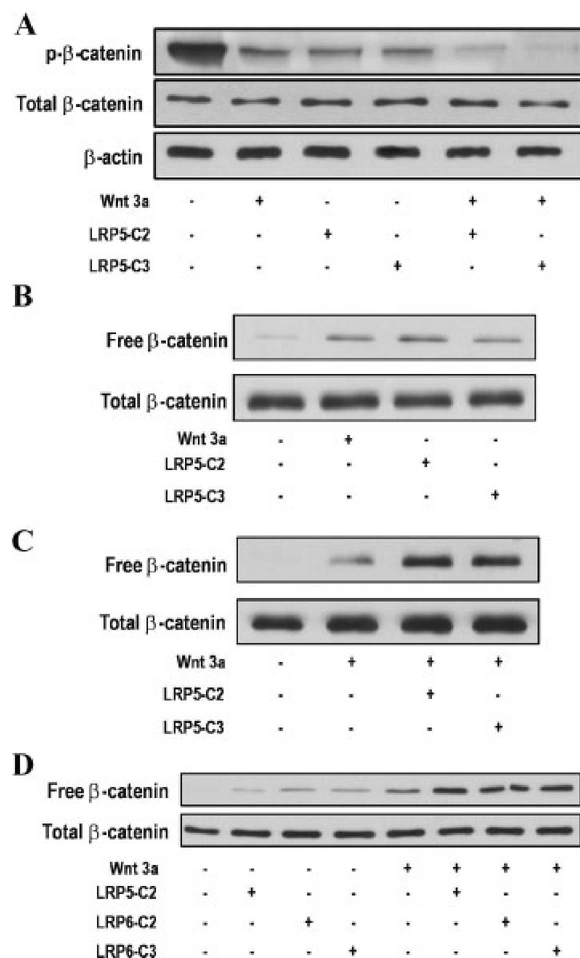

Fig. 5. Both LRP5-C2 and LRP5-C3 decreased the phosphorylation of β-catenin and increased the levels of free cytosolic β-catenin. COS-7 cells were transiently transfected with 0.5 μg LRP5/6-C2, LRP5-C3, or vector only (−), as indicated, for 24 h prior to treatment with LCM (-Wnt 3a) or Wnt 3aCM (+Wnt 3a). **A**: Cells were treated with LCM or Wnt 3aCM for 2 h and cytosolic fractions (30 μg) were probed for endogenous phospho-β-catenin (Ser33/37/Thr41; p-β-catenin). Compared to the LCM treated cells, the phospho-β-catenin levels in both LRP5-C2 and LRP5-C3 transfected cells were decreased and to levels that were similar to those observed in cells treated with just Wnt 3aCM. Treatment of LRP5-C2 and LRP5-C3 transfected cells with Wnt 3aCM resulted in a further decrease in the levels of phospho-β-catenin compared to what was observed in cells treated with Wnt 3aCM alone. Total β-catenin levels were unchanged. β-actin was used as a loading control. **B**: Cells were treated with LCM or Wnt 3aCM for 1h. Free β-catenin was pulled down from 25 μg total cell lysate using pGST-E-cadherin. Expression of LRP5-C2 or LRP5-C3 increased free β-catenin levels relative to vector transfected cells, and was comparable to the levels in Wnt 3aCM treated cells. Total β-catenin (2 μg) levels did not change. **C**: Cells were treated with LCM or Wnt 3aCM for 4 h. LRP5-C2 and LRP5-C3 potentiated the increase in free β-catenin levels induced by treatment with Wnt 3aCM. Total β-catenin (2 μg) levels were unchanged. **D**: Cells were treated with LCM or Wnt 3aCM for 4 h. LRP6-C2 and LRP6-C3 potentiated the increase in free β-catenin levels induced by treatment with Wnt 3aCM, which is comparable to LRP5-C2. Total β-catenin (2 μg) levels did not change.

respect to the events that occur at upstream sites, especially at the cell membrane. Wnt proteins have been shown to bind to Fz receptors within an N-terminal cysteine-rich-domain (CRD) [Bhanot et al., 1996; Yang-Snyder et al., 1996]. Further, previous reports have provided solid evidence for the involvement of LRP5/6 in the Wnt canonical pathway in *Drosophila, Xenopus*, and mice [Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000]. LRP5 and LRP6 are the only two proteins from LDL-receptor family that have been shown to facilitate signaling through the Wnt canonical pathway, although a minireceptor of LRP1 has been shown to repress Wnt 3a signaling [Zilberberg et al., 2004]. In this present study only LRP6 stimulated the TCF/LEF-1 activation in the absence of extracellular Wnt 3a. In support of our findings a previous study showed that LRP6 but not LRP5 resulted in axis duplication in *Xenopus*, suggesting that only LRP6 in the absence of Wnt can stimulate the β-catenin pathway [Tamai et al., 2000]. Further, it was shown that LRP6 alone increased LEF-1 activity in *Drosophila* S2 cells, however LRP5 was not examined in this study [Schweizer and Varmus, 2003]. These data suggest that LRP5 and LRP6 play different roles in modulating Wnt canonical pathway, beyond differential binding to the various Wnts. Given the fact that the extracellular domains of LRP5 and LRP6 differ, it can be postulated that an endogenous inhibitor (such as Dickkopf1 [Zorn, 2001]) may preferentially bind LRP5 and thus the presence of Wnt may be required to relieve the inhibition on LRP5-mediated TCF/LEF-1 signaling. Nonetheless, both LRP5 and LRP6 strongly potentiate Wnt 3a-induced activation of the Wnt canonical pathway, confirming previous studies [Tamai et al., 2000; Gong et al., 2001; Caricasole et al., 2003].

Expression of the cytoplasmic domain of LRP5 or LRP6, with or without the transmembrane domain, resulted in a down regulation of cytosolic phospho-β-catenin levels, an increase in free β-catenin levels and constitutive TCF/LEF-1 activation. The finding that the intracellular domains of LRP5 and LPR6 activated TCF/LEF-1 to the same extent suggests that the cytoplasmic domains of LRP5 and LRP6 play a similar role in the activation of TCF/LEF-1. Intriguingly, membrane localization did not affect the ability of the cytoplasmic domains of LRP5 and LRP6 to activate TCF/LEF-1, in contrast to the findings of a previous report [Mao et al., 2001]. In this previous study, it was shown that a construct containing the intracellular domain and the transmembrane region of LRP5 was significantly more active than one containing just the intracellular domain although both were constitutive activators of LEF-1 [Mao et al., 2001], as we also observed. It was suggested that this was because the membrane anchored form of the intracellular domain LRP5 facilitated the recruitment of axin to the membrane where it was degraded thus resulting in increased levels of β-catenin [Mao et al., 2001]. It should be noted that detectable axin degradation occurs several hours after Wnt stimulation, while β-catenin stabilization is detectable within 30 min [Willert et al., 1999], although it has been postulated that only a small reduction in axin is needed to have a significant effect on β-catenin levels (for a review see [He et al., 2004]). Nonetheless it needs to be considered that the recruitment of axin to the membrane followed by degradation of axin is likely only one mechanism by which LRP5 and LRP6 facilitate β-catenin stabilization and TCF/LEF-1 signaling. Our data clearly demonstrate that the intracellular domains of LRP5 and LRP6 without the transmembrane domains are very effective at potentiating Wnt signaling. Although the mechanism by which the intracellular domains of LRP5 and LRP6 activate TCF/LEF-1 and potentiate Wnt signaling is unknown, it is clearly independent of direct membrane association. Possible mechanisms by which the intracellular domains of LRP5 and LRP6 facilitate Wnt canonical signaling may involve such events as inhibition of casein kinase Iα which primes β-catenin for phosphorylation by GSK3β resulting in β-catenin degradation [Liu et al., 2002], facilitation of Dsh activation [Sun et al., 2001] and dissociation of GSK3 from the axin-APC-β-catenin complex (for a review see [He et al., 2004]). It is also possible that the intracellular domains of LRP5 and LRP6 may still associate with the intracellular domains Fz receptors and thus "prime" the signaling pathway. The finding that the intracellular domains of LRP5 and LRP6 strongly potentiate Wnt 3a-induced activation of TCF/LEF-1 was quite surprising given the fact that previous reports have suggested that the extracellular domain of LRP6 is required for the potentiation of Wnt-induced LEF activation through the Fz8 receptor [Tamai et al., 2000].

Interesting LRP5/6-C2 and LRP5/6-C3 potentiated Wnt 3a-induced TCF/LEF-1 activation to a greater extent than FL-LRP5/6. These findings suggest that the intracellular domain of LRP5 and LRP6 play an essential role in regulating the Wnt signaling, independent of effects on ligand binding. The mechanisms involved are currently unknown and under investigation.

In conclusion the results of these studies demonstrate that in the absence of exogenously added Wnt 3a LRP6, but not LRP5, results in TCF/LEF-1 activation. Further, we show that expression of the intracellular domain of LRP5 or LRP6, both membrane-anchored and cytosolic, results in activation of the Wnt signaling pathway. In addition, both LRP5/6-C2 and LRP5/6-C3 greatly potentiated Wnt signaling. These findings indicate that membrane-anchoring is not essential for the activation of TCF/LEF-1 by LRP5 and LRP6. Investigations are currently underway to elucidate the mechanisms by the intracellular domain or LRP5/6 regulates the Wnt signaling pathway.

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