Second Cysteine-rich Domain of Dickkopf-2 Activates Canonical Wnt Signaling Pathway via LRP-6 Independently of Dishevelled*

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Wnt family of secretory glycoproteins is one of the major families of developmentally important signaling molecules and plays important roles in embryonic induction, generation of cell polarity, and specification of cell fate (1–3). Wnt pathways are also closely linked to tumorigenesis (3–5). Studies using Drosophila, Xenopus, and mammalian cells have established a canonical signaling pathway. Wnt proteins bind cell surface receptors Frizzled (Fz) and LDL-receptor related protein (LRP)-5/6 and prevent glycogen synthase kinase 3 (GSK3)-dependent phosphorylation of β-catenin, thus leading to the stabilization of β-catenin. The stabilized β-catenin interacts with transcription regulators, including lymphoid-enhancing factor-1 (LEF-1) and T cell factors, and activates gene transcription (2, 6, 7).

Both genetic and biochemical results have provided solid evidence indicating that Fz proteins function as Wnt receptors (2). Recently, LRP-5 and LRP-6, members of the LDL receptor superfamily, were found also to be required for the canonical Wnt signaling pathway. A Drosophila mutant arrow, which encodes a close homolog of LRP-5/6, shows phenotypes similar to the wg mutant (8). In addition, mammalian LRP-6 was shown to bind to Wnt-1 and enhance Wnt-induced developmental processes in Xenopus embryos (9). Moreover, mice lacking LRP-6 exhibited developmental defects that are similar to those caused by deficiencies in various Wnt proteins (10). Our recent work revealed how LRP-5/6 might be involved in transducing signals. We found that Wnt proteins induce the binding of LRP-5 to Axin, leading to Axin degradation and β-catenin stabilization (11).

Xenopus Dickkopf (Dkk)-1 was initially discovered as a Wnt antagonist that plays an important role in head formation (12). By far, four members of Dkk have been identified in mammals (13, 14). Each Dkk molecule contains two conserved cysteine-rich domains as depicted in Fig. 1A. The mechanism by which the Dkk family of proteins antagonizes Wnt effects was not known until recently. Several recent reports showed that Dkk-1 and Dkk-2 inhibited the canonical Wnt signaling by binding to LRP6 in cultured mammalian cells (15–17). However, Dkk-2 has also been shown to activate the Wnt/β-catenin pathway in Xenopus embryos (18). To gain an understanding of this paradoxical phenomena and insight into the interactions between Dkk and LRP proteins, we examined the effect of each cysteine-rich domains of Dkk-1 and Dkk-2 on the canonical Wnt signaling in mammalian cells. We found that the second Cys-rich domains of Dkk1 and Dkk2 inhibited Wnt-3a-activated signaling, whereas the first Cys-rich domains had no

The abbreviations used are: LRP, LDL-related protein; GSK, glycogen synthase kinase; LEF, lymphoid-enhancing factor; LDL, low density lipoprotein; Dkk, Dickkopf; GFP, green fluorescent protein; siRNAs, short interfering RNAs; CM, conditioned medium.
effects. Intriguingly, the second Cys-rich domain of Dkk-2, but not that of Dkk-1, was able to activate the canonical pathway in the presence of LRP6, and this LRP-dependent signaling does not require Dvl.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, Preparation of Conditioned Medium, and Luciferase Assay**—Human embryonic kidney cell line A293 and mouse fibroblast cell line NIH 3T3 were maintained and transfected as previously described (21). For luciferase assays, NIH3T3 cells in 24-well plates were seeded at 5 × 10^4 cells/well and transfected with 0.5 μg of DNA/well using LipofectAMINE Plus (Invitrogen, Carlsbad, CA), as suggested by the manufacturer. Cell extracts were collected 24 h after transfection. Luciferase assays were performed as previously described (20, 21). Luminescence intensity was normalized against fluorescence intensity of GFP. For preparation of Dkk containing conditioned medium (CM), A293 cells were seeded in 6-well plates at 4 × 10^5 cells/well and transfected with 1 μg of DNA/well. CMs were collected 48 h after transfection. Wnt-3a-containing CM was prepared as previously described (11, 28).

**Construction of Expression Plasmids and Mutagenesis**—The wild-type and mutant forms of human LRP-5, LRP-6, mouse Wnt-1, Dvl-1, GSK-3β, and Axin were generated by PCR using the high fidelity thermostable DNA polymerase Pfu (Stratagene, La Jolla, CA). Myc, hemagglutinin, or FLAG epitope tags were introduced to the C termini of the full-length and mutant molecules. The expression of these molecules was driven by a cytomegalovirus promoter (20–22). All constructs were verified by DNA sequencing. The LEF-1 reporter gene constructs were kindly provided by Dr. Grassé (19).

**Preparation of Anti-LRP Antibody and Dvl-specific Short Interfering RNAs (siRNAs)**—The anti-LRP5/6 antibody was generated by A&G Pharmaceuticals (Baltimore, MD) using a synthetic peptide derived from the amino acid sequence (DTGTDRIEVTR) of the second YWTD repeat of LRP-6. The siRNA targeted both human Dvl-1 and Dvl-3 are derived from an mRNA sequence (AACAAGATCACCTTCTCCGAG) of human Dvl-3, which is identical in that of human Dvl-1, and the one targeted human Dvl-2 is derived from an mRNA sequence (AACTTT-GAGAACATGAGCAAC) of human Dvl-2. The siRNA duplexes were prepared by Dharmaco Research (Lafayette, CO). Transfection of siRNA was performed based on a protocol provided by Dharmaco Research using Oligofectamine (Invitrogen).

**RESULTS**

To investigate the effect of each Cys-rich domain of Dkk-1 and Dkk-2 on the canonical Wnt signaling pathway, we generated a number of plasmids expressing either full-length or individual Cys-rich domains of Dkk-1 and Dkk-2 as depicted in Fig. 1A. CM generated in A293 cells was analyzed by Western blotting using a monoclonal antibody specific to the FLAG tag. All of the Dkk molecules were expressed with a FLAG-tag. The multiple bands observed in some of the lanes may result from proteolysis or post-translational modification.

**Fig. 1. Preparation of CM containing Dkk and its mutants.** A, schematic representation of full-length Dkk-1 and Dkk-2 and their mutants. The Cys-rich domains are shaded, and the number of residues for each molecule is noted. B, Western analysis of CM and cell lysates containing Dkk and its mutants. Eight microliters of CM were mixed with 2 μl of the SDS-PAGE sample buffer and analyzed by Western blotting with an antibody specific to the FLAG tag. For preparation of whole cell lysates, cells were directly lysed by the SDS-PAGE sample buffer, and the same amounts of samples were analyzed by Western blotting. All of the Dkk molecules were expressed with a FLAG-tag. The activity of each Cys-rich domain of Dkk-1 and Dkk-2 on the canonical Wnt signaling was determined by examining the LEF-1-dependent transcriptional activity using a luciferase reporter gene assay. Numerous difficulties have been published that the Wnt-1 class of Wnt proteins, which also includes Wnt-3a, activates LEF-1-dependent transcription via stabilization of β-catenin (11, 19–22). We found that none of CMs alone showed any effect on LEF-1-dependent transcription when CMs were added to NIH3T3 cells transfected with the LEF-1-dependent reporter gene (Fig. 2A). CM containing Wnt-3a was able to activate LEF-1 in the same system (Fig. 2A) as we have previously shown (11). When CM containing a Dkk protein or one of the Cys-rich domains was added together with CM containing Wnt-3a, the full-length Dkk-1 and Dkk-2 as well as the second Cys-rich domains of both Dkk-1 and Dkk-2 inhibited Wnt-3a-induced activation of LEF-1 (Fig. 2A). The inhibitory effects of full-length Dkk proteins seen here are consistent with those previously reported (15–17). The fact that neither of the first Cys-rich domains of Dkk-1 and Dkk-2 inhibited the Wnt-3a effect (Fig. 2A) suggests
LEF-1 expression plasmid, 0.075 g of a LEF-1 expression plasmid, 0.075 g of a LEF-1 luciferase reporter plasmid, 0.15 g of a GFP expression plasmid, and 0.15 g of LacZ, LRP-5 (A), or LRP-6 expression plasmids (A and B). The assay and data processing were carried out as described in Fig. 2A.

that the second cysteine-rich domain mediates the inhibitory effect of Dkk molecules.

Previous studies on Dkk proteins (15–17) found that overexpression of LRP-6 was able to attenuate the inhibitory effects of the full-length Dkk proteins. Consistent with these findings, we found that expression of LRP-6 alleviated the inhibition by the full-length Dkk-1 or Dkk-2 (Fig. 2B). Although the expression of LRP-6 also abolished the inhibition caused by Dkk-1C2, we were surprised to find that Dkk-2C2 activated LEF-1 in the presence of LRP-6 and that there appeared to be an additive effect between Dkk-2C2 and Wnt-3a in activation of LEF-1 (Fig. 2B). We also examined the effect of LRP-5; Dkk-2C2 showed a marginal effect on LEF-1 activation in the presence of LRP-5 (Fig. 3A).

To gain a better look at this Dkk-2C2-mediated effect on the canonical Wnt signaling, we examined the dose-dependence by serial dilution of CM containing Dkk-2C2 in comparison to CM containing Wnt-3a. The maximal activation (about 4-fold) was reached at 1:4 dilution (Fig. 3B). Addition of more CM containing Dkk-2C2 appeared to cause a decline in the activity, indicating a bell-shaped dose-dependence. In comparison, the Wnt3a CM showed a higher activity than the Dkk-2C2 CM and did not exhibit the bell-shape dose dependence (Fig. 3B).

To understand how Dkk-2C2 activates the canonical Wnt signaling pathway, we first wanted to determine whether the signaling events induced by Dkk-2C2 are mediated by LRP-6. We obtained a monoclonal antibody kindly provided by A&G Pharmaceuticals raised against a synthetic peptide derived from the second YWTD repeat of human LRP-6. These residues are completely conserved between human and mouse and between LRP-5 and LRP-6. Western analysis using this monoclonal antibody showed that the antibody detected two bands in the particulate fraction prepared from NIH3T3 cells, and both bands were intensified in the particulate fraction sample from cells transfected with LRP-6 (Fig. 4A). In addition, neither band was detected in the soluble fractions (Fig. 4A). The upper band with an apparent molecular mass larger than 175 kDa appears to be the full-length LRP-6, whereas the exact nature of the lower molecular mass band is not clear, which may be a proteolytic fragment LRP-6. The antibody was tested for neutralizing the activity induced by Wnt-3a or Dkk-2C2. As shown in Fig. 4B, the anti-LRP antibody significantly inhibited Dkk-2C2, and Wnt-3a-induced activation of LEF-1 by 80 and 50%, respectively. The antibody showed little effect on β-catenin or Dvl-induced activation of LEF-1 (Fig. 4B). This result suggests that both Wnt-3a and Dkk-2C2 signal through LRP-6 and its homologs.

We have previously shown that interactions of LRP-5/6 with Axin are involved in LRP-mediated signaling and that expression of Axin could block the activity of an activated form of LRP-5, LRPC2 (15). We tested if Axin would block Dkk-2C2-mediated activation of LEF-1. Fig. 5A shows that expression of Axin blocked Dkk-2C2’s effect. Previous studies have also suggested that Dvl might act downstream of LRP on the basis of the observation that the DIX domain of Dvl could inhibit LRP-mediated effects (9). We also observed an inhibitory effect of Dvl DIX domain on LEF-1 activation by Dkk-2C2 (Fig. 5A) or LRPC2 (Fig. 5B). However, because the DIX domain of Axin is required for the interaction of Axin with LRPC2 (11) and Axin DIX interacts with Dvl DIX (22), overexpression of Dvl DIX may inhibit LRPC2 by interfering the interaction of LRPC2 with Axin. To more definitively evaluate the involvement of Dvl in LRP signaling, we used another previously defined Dvl-derived dominant negative mutant, the Dvl DEP domain. It was shown to inhibit Wnt signaling by interacting with upstream regulators of Dvl (23). Although Dvl DEP inhibited Wnt-3a-induced activation of LEF-1 (Fig. 5D), it showed no effect on Dkk-2C2- or LRPC2-induced activation of LEF-1 (Fig. 5, A and B). In addition, we found that GSK, which inhibits both Wnt and Dvl-induced LEF-1 activation (Fig. 5, C and D) (24), had little effect on the activation of LEF-1 by either Dkk-2C2 or LRPC2. We interpret these results to suggest that Dvl may not be required for LRP-mediated canonical Wnt signaling.

To further confirm the idea that Dvl may not be involved in LRP-mediated signaling, we employed a newly developed technique, short interfering RNA (siRNA) (25), to knock down the Dvl expression. Two siRNAs consisting of 21-mer duplexed RNA were designed; one is based on a nucleotide sequence shared by human Dvl-1 and Dvl-3, and the other is based on a sequence from human Dvl-2 that has one nucleotide mismatch from Dvl-3. To examine the efficacy and specificity of this siRNA-mediated knocking-down approach, we examined the levels of Dvl-3 proteins in A293 cells treated with or without siRNAs by Western analysis using a monoclonal antibody specific to Dvl-3. The Dvl-3 protein can be readily detected in these cells by this antibody (Fig. 6A). The siRNA specific to Dvl1 and
cells in 24-well plates were transfected with 0.025 \( \mu \text{g} \) of a LRP-6 expression plasmid, 0.075 \( \mu \text{g} \) of a LEF-1 luciferase reporter plasmid, 0.15 \( \mu \text{g} \) of a GFP expression plasmid, and 0.15 \( \mu \text{g} \) of a LacZ expression plasmid. 1 day later, the cells were transfected with both siRNAs and 2 days later with the LEF-1 reporter gene, Dvl (C), or LacZ (D) expression plasmid and 0.15 \( \mu \text{g} \) of a LacZ, Axin, Dvl DX, Dvl DEP, or GFP expression plasmid. Control CM or CM containing Dkk-2C2 (A) or Wnt-3a (D) was added before the luciferase assay.

**Figure 5.** Delineation of signaling pathways for Dkk-2C2. NIH3T3 cells in 24-well plates were transfected with 0.025 \( \mu \text{g} \) of a LEF-1 expression plasmid, 0.075 \( \mu \text{g} \) of a LEF-1 luciferase reporter plasmid, 0.15 \( \mu \text{g} \) of a GFP expression plasmid, 0.15 \( \mu \text{g} \) of a LRP-6 (A), LRPC2 (B), Dvl (C), or LacZ (D) expression plasmid and 0.15 \( \mu \text{g} \) of a LacZ, Axin, Dvl DX, Dvl DEP, or GFP expression plasmid. Control CM or CM containing Dkk-2C2 (A) or Wnt-3a (D) was added before the luciferase assay.

Dvl-3 could knock down the protein level of Dvl-3 by approximately 70% after a three-day treatment, whereas the siRNA specific to Dvl-2 also showed some effect on Dvl-3 expression; an approximate 15% inhibition. We also detected the levels of Actin as an internal control to exclude potential variations introduced by siRNA treatment or sample loading (Fig. 6 A). These results indicate that this knockdown approach yields a reasonable efficacy and specificity. To test the effect of these siRNAs on LRP-1 activity, A293 cells were first transfected with siRNAs and 2 days later with the LEF reporter gene and Wnt3a or LRPC2. These Dvl-specific siRNA molecules inhibited Wnt-3a-induced LEF-1 activation by close to 70%, whereas they had no effects on LRPC2-induced activation of LEF-1 (Fig. 6). This result corroborates our conclusion that Dvl may not play a role in LRP-mediated LEF-1 activation.

**Figure 6.** Role of Dvl in LRP-mediated signaling. A) AS293 cells were transfected with no siRNA (Lane 1), a siRNA specific to Dvl-2, a siRNA specific to both Dvl-1 and Dvl-3 or both siRNAs. Cells were analyzed by Western blotting with a monoclonal antibody specific to Dvl-3 (upper panel) or to Actin (lower panel) after 3 days. B, A293 cells were transfected with both siRNAs and 2 days later with the LEF-1 reporter gene, GFP, and LacZ, LRPC2, or Wnt-3a expression plasmids.

**DISCUSSION**

Previous reports have demonstrated that Dkk-1 and Dkk-2 are antagonists of Wnt proteins that activate the canonical pathway in mammalian cells (15–17). In this report, we describe that the second Cys-rich domain of Dkk-2, but not that of Dkk-1, can act as an agonist for the canonical Wnt signaling pathway in mammalian cells. More importantly, we present evidence indicating that Dkk-2C2 functions through LRP-6 and that this LRP-mediated signaling may not require Dvl. Thus, it appears that LRP and Fz may utilize distinct mechanisms for their immediate signaling events. Although the mechanisms by which Fz regulates its immediate intracellular signaling components are not clear, one mechanism for LRP may be mediated by the interaction with and destabilization of Axin (11).

It is apparent that Dkk-2C2 uses only LRP in its signal transduction while Wnt-3a uses both LRP and Fz in its activation of LEF-1 as depicted in Fig. 7. Several lines of evidence support this idea. 1) Genetic studies in *Drosophila* have clearly demonstrated that both Fz (26, 27) and LRP (8) are required for the signaling of Wg, the fly homolog of Wnt-1. 2) The LRP-specific antibody blocked activity of both Wnt and Dkk-2C2 (Fig. 4B), suggesting both Wnt and Dkk-2C2 require LRP for their signaling. The apparent low potency of this antibody in neutralizing Wnt-3a may be due to its low affinity for other YWTD repeat sequences (data not shown) that may be involved in Wnt recognition or its low neutralizing activity. 3) While Dvl has been strictly identified as a downstream effector for Fz by epistasis analysis in the study of *Drosophila* (1–3), we showed that Dvl is not required for Dkk-2C2 and LRP-mediated activation of LEF-1 because GSK (a potent inhibitor of Dvl and Fz) and Dvl DEP could not inhibit the activation of LEF-1 by Dkk-2C2 or by an activated form of LRP, LRPC2 (Fig. 5). The finding that knocking down the expression of Dvl only inhibited Wnt-induced activity, but not LRPC2-induced activity, (Fig. 6) further supports the conclusion that Dvl may not be required for Dkk-2C2 and LRP-mediated signaling. Although it was shown that expression of wild-type Dvl could rescue an arrow mutant (arrow is the fly homolog of LRP-5/6) (8), the complete epistatic relationship between Arrow and Dvl needs to be established by showing that hypomorphic or null dsh alleles can suppress the activity of an activated form of LRP or the overexpression of wild-type LRP. 4) Dkk proteins were shown to bind to LRP proteins, but not Fz proteins (15, 16). However, there is an apparent discrepancy in describing the binding of Dkk-2 to LRP-5. Our measurement using a fusion protein of Dkk-2 and alkaline phosphatase suggests that Dkk-2C2 can bind to LRP-5, but with a significant lower affinity than that for LRP-6 (data not shown). The low affinity for LRP-5 may explain the low activity of Dkk-2C2 in the presence of LRP-5 (Fig. 3A). 5) Dkk-2C2 activated the canonical pathway only when LRP-6 was overexpressed, suggesting a dependence on LRP-6 for its activity. This also suggests that Dkk-2C2 only signals when a sufficient amount of LRP-6 is present and that NIH3T3 cells may not contain enough endogenous LRP-6 for Dkk-2C2 to
function despite that we can detect LRP-6 in these cells by both Western (Fig. 4A) and reverse transcription-PCR (data not shown).

It seems to be paradoxical that Dkk-2C2 inhibits Wnt’s effect in the absence of LRP-6, while it acted additively with Wnt in activation of LEF-1 in the presence of LRP-6 (Fig. 2). We would explain this apparent paradox to suggest that Wnt is a more potent ligand for activating the canonical pathway than Dkk-2C2. The dose-dependence study (Fig. 3B) supports this point. In addition, we also noticed that Dkk-2C2 showed a bell-shape dose-dependent curve, suggesting that Dkk-2C2’s activity declines at higher doses. Thus, it is not difficult to understand that the relative inefficiency of Dkk-2C2 in activating the canonical pathway would become antagonistic to Wnt by competing for binding to the limited number of endogenous LRP required for Wnt to signal. The high efficiency of Wnt-activated signaling that is enabled by the utilization of both LRP and Dvl-mediated pathway as proposed in the model shown in Fig. 7 may be required for many Wnt-regulated biological processes, which may include fly embryonic development where disruption of either pathway led to the similar phenotypic outcomes.

Our finding that Dkk-2C2 can activate the canonical pathway may also help to understand the paradox that although both Dkk-1 and Dkk-2, when transfected in mammalian cells, inhibited the canonical Wnt activity, only Dkk-1 apparently antagonized the canonical pathway in Xenopus. Dkk-2, when its RNA was injected into Xenopus embryos, activated the canonical pathway (18). It is possible that Dkk-2 is proteolytically processed in Xenopus embryos, thus producing Dkk-2C2-like molecules that can activate the canonical pathway. Alternatively, accessory proteins may exist in these embryos, presenting Dkk-2 in an agonistic form. Therefore, it would be of great interest to determine whether Dkk-2C2-like molecules exist naturally and to understand the physiological significance of their existence. Nevertheless, even if Dkk-2C2-like molecules do not exist naturally, they are useful tools for studying LRP-6-mediated signaling events.

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