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Analysis of Dickkopf3 interactions with Wnt signaling receptors

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Abstract

Wnt signaling regulates essential biological processes ranging from embryogenesis to neurodegeneration. Recently, we demonstrated that Dickkopf3 (Dkk3) is a pro-survival glycoprotein that positively modulates Wnt signaling. An important step in understanding the mechanism of action of Dkk3 is identifying its interacting proteins in the Wnt pathway. In this study, we used a series of biochemical and functional assays to investigate the interaction between Dkk3 and the Wnt pathway receptors Kremen1 (Krm1), Kremen 2 (Krm2) and low-density lipoprotein receptor-related protein 6 (LRP6). Here, we report that, contrary to previous studies, Dkk3 interacts with Krm1 and Krm2. However, Dkk3 did not interact with, or alter expression of, LRP6. Blocking protein glycosylation did not alter the interaction between Dkk3 and Krm proteins. Additionally, Krm2 abolished Dkk3-mediated potentiation of Wnt signaling. Therefore, our data establish that Krm proteins are novel binding partners of Dkk3 and suggest a mechanism by which Dkk3 potentiates Wnt signaling.

Keywords

Wnt signaling pathway; Dickkopf3; Kremen; immunoprecipitation

Introduction

Wnt signaling pathways regulate a wide range of essential processes in embryonic and adult tissues (De Ferrari and Moon 2006). The best characterized Wnt signaling pathway is the canonical/Wnt- β -catenin pathway. In the absence of Wnt ligands, the adenomatous polyposis coli/Axin/glycogen synthetase kinase 3 β (GSK3 β) kinase complex phosphorylates the central effector β -catenin, resulting in its degradation by the proteasome and suppression of β -catenin levels. Binding of Wnt ligands to the cell surface co-receptors Frizzled and lipoprotein receptor-related protein 5/6 (LRP5/6) leads to disruption of GSK3 β kinase activity. As a result, β -catenin is stabilized and translocates into the nucleus, where it binds with T-cell factor/lymphoid-enhancing binding factor transcription factors to drive expression of Wnt target genes.

The Dickkopf (Dkk) family, composed of Dkk1, 2, 3, and 4 and soggy, is a group of secreted glycoproteins that regulate the Wnt pathway. Dkk proteins have been implicated in various diseases, including retinal degeneration (Hackam et al. 2004), malignancies (Hsieh et al. 2004), Alzheimer's disease (Alvarez et al. 2004) and cerebral ischemia (Mastroiacovo et al. 2009). One of the least characterized members of the Dkk family is Dkk3. Recent work demonstrated that Dkk3 has distinct roles in regulating the Wnt pathway depending on the cell

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types examined. For example, Dkk3 potentiates Wnt signaling in human Muller glia cell MIO-M1 and HEK293 cell lines (Nakamura et al. 2007), but inhibits Wnt signaling in PC12 and osteocarcinoma Saos-2 cells (Caricasole et al. 2003; Hoang et al. 2004). Dkk3 also reduces cell death in HEK293 cells exposed to hydrogen peroxide and staurosporine (Nakamura et al. 2007) and protects osteocarcinoma cells against apoptosis caused by chemotherapy agents and serum starvation (Hoang et al. 2004), but induces apoptosis in liver cancer cell lines (Hsieh et al. 2004) and prostate cancer cell lines (Abarzua et al. 2005). The molecular basis of Dkk3 activity in Wnt signaling and apoptosis is unknown.

The interaction between Dkk1, Dkk2, and Dkk4 with the Wnt co-receptors LRP5/6 and Kremen (Krm) has been reported previously (Davidson et al. 2002; Mao et al. 2002). The formation of a Dkk1, Krm, and LRP trimer protein complex inhibits Wnt signaling by internalization of the receptor complex, which depletes cell surface expression of LRP (Mao et al. 2002; Yamamoto et al. 2006, 2008; Wang et al. 2008). Despite the high sequence homology between Dkk3 and Dkk1, interacting proteins of Dkk3 in the Wnt pathway have not been identified. Extracellular Dkk3 did not bind to surface-expressed Krm1 and Krm2 proteins in HEK293 cells (Mao et al. 2002). Furthermore, Dkk3 did not compete against Dkk1 binding to Krm proteins (Mao et al. 2002). Therefore, in the current study, we used a combination of biochemical and functional assays to further characterize interactions between Dkk3 and the Wnt co-receptors Krm1, Krm2, and LRP6. Our analyses indicated that Dkk3 interacts with Krm1 and Krm2, and that this interaction was not affected by the inhibition of glycosylation. Furthermore, Krm2 but not Krm1 significantly reduced the Dkk3-mediated potentiation of Wnt signaling. These data demonstrate novel molecular interactions among Dkk3, Krm1, and Krm2 and provide insight into the mechanism of action of Dkk3 in the Wnt signaling pathway.

Material and methods

Cell culture and reagents

The SH-SY5Y and HEK293 cell lines were used because they are Wnt-responsive and have high transfection efficiency. Both cell lines were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum, 1% penicillin and streptomycin, and 10 μ g/ml l-glutamate. The establishment and maintenance of an HEK293 cell line stably expressing Dkk3, and the control line transfected with the parental vector, were described previously (Nakamura et al. 2007). FLAG-epitope-tagged mouse Dkk1 and mouse Dkk3, V5-tagged mouse Krm1 and mouse Krm2, and human LRP6 expression vectors were generously provided by Dr C. Niehrs (Heidelberg, Germany). The TOP-FLASH luciferase reporter plasmid was a generous gift from Dr R. Moon (HHMI, University of Washington). Canonical Wnt signaling was induced using conditioned media (CM) prepared from mouse L-cells stably expressing Wnt3a (ATCC, Manassas, VA, USA) that had been filtered and mixed 1:1 with normal media for use. The control CM was prepared from parental L-cells. Dkk3-containing CM was prepared from confluent HEK293 cultures stably expressing Dkk3.

Glutamate-S transferase-pull down assays and Western blot analysis

Dkk1 and Dkk3 sequences containing a partial cysteine-rich domain (CRD1) and the full CRD2 domain followed by the FLAG (DYKDDDDK) sequence from nucleotides 251 to 843 for Dkk1 and 1527–1149 for Dkk3 were subcloned into the Glutamate-S transferase (GST) expression pGEX-2T vector using *Eco* RI restriction sites for Dkk1 and Dkk3. Subcloned plasmids were transformed into BL21 *Escherichia coli* bacteria (Promega, Madison, WI, USA). GST-fusion proteins were induced using 200 μ M isopropyl- β -D-1-thiogalactopyranoside (Gold Biotechnology, St Louis, MO, USA) for 4 h, which was determined to be the optimal concentration and time. The bacteria pellet was sonicated in cold phosphate buffered saline with 1% triton-X containing the proteinase inhibitor 1% phenylmethanesulfonylfluoride

(Roche, Basel, Switzerland). The bacterial lysate was cleared by centrifugation at 10,000 rpm, and the supernatant containing fusion protein was purified using B4 sepharose beads (Thermo, Rockford, IL, USA), overnight at 4°C. Fusion protein bound to beads was resolved using 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels followed by densitometric analysis of the Coomassie-stained gel to quantify the purified protein. Specific concentrations of bovine serum albumen (Sigma, St Louis, MO, USA) were used as a standard. Approximately 10 µg of fusion protein was incubated with 100 µg of SH-SY5Y lysate prepared from cells overexpressing either V5-tagged Krm1 or Krm2. After overnight incubation at 4°C, the protein complexes were washed with lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% EDTA, 1% NP40) and electrophoresed on a SDS-PAGE gel using Tris-glycine buffer. Proteins were then transferred onto polyvinylidene fluoride membranes, and were probed with anti-V5 (Sigma) antibodies followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), followed by incubation with enhanced chemiluminescence reagent-plus (GE Amersham, Piscataway, NJ, USA). The antibody dilutions used are listed in Table I.

Immunoprecipitation

Approximately 100,000 SH-SY5Y cells were co-transfected with Dkk1, Dkk3, Krm1 or Krm2 using Lipofectamine2000 reagent (Invitrogen, Carlsbad, CA, USA) with a ratio of 1 µg of plasmid to 4 µl of lipid. After 48 h, the cells were harvested and soluble protein was extracted using 300 µl of lysis buffer with proteinase inhibitors (Complete, Roche, Basel, Switzerland). Ten microliters of lysate was saved for later analysis and the remainder was used for co-immunoprecipitation (IP) with beads linked to the M2 antibody that recognizes the FLAG tag (Sigma). After an overnight incubation at 4°C, the protein complexes attached to the M2 beads were washed with lysis buffer three times and eluted with 80 µl of 3X FLAG peptide (Sigma). Twenty microliters of elution and 10 µl of original lysate were analyzed by Western blotting. An antibody against the V5-epitope was used to test Dkk3 interaction with Krm1 or Krm2, and an antibody against the FLAG-epitope was used as an IP control.

For the LRP6 co-IP experiment, SH-SY5Y cells were co-transfected with LRP6 and Dkk plasmids. Transfected cells were treated with control CM or Wnt3a containing CM. Whole cell lysate and media were immunoprecipitated with anti-FLAG antibody followed by Western blot analysis using antibodies against FLAG-epitope and LRP6, as described above.

Subcellular fractionation was performed using the Proteoextract Subcellular Proteome kit (Calbiochem, Gibbstown, NJ, USA) according to the manufacturer's protocol. The cell pellet was fractionated into cytoplasmic (C), membrane (M), and nuclear (N) fractions. Protein content was quantified using the Lowry method (BioRad, Hercules, CA, USA). Twenty micrograms of each fraction were resolved in 8% or 10% gels followed by Western blotting analysis.

Confocal analysis was performed using our previously published procedures for immunohistochemistry (Nakamura et al. 2007). The images were obtained and processed using a Leica TCS SP5 confocal microscope.

Glycosylation analysis

Whole cell lysates were obtained from SH-SY5Y cells transfected with V5-Krm1, V5-Krm2, or LRP6 expression plasmids. Twenty micrograms of lysate protein were subjected to peptide N-glycosidase F (PNGF) treatment (New England Bioscience, Ipswich, MA, USA) following the manufacture's protocol or left untreated. After treatment, the lysates were analyzed by Western blotting using antibodies against LRP6 or the V5 epitope. Additionally, SH-SY5Y cells were transfected with FLAG-Dkk3 and V5-Krm1 or V5-Krm2 plasmids and at 24 h after transfection fresh media were added containing 1 µg/ml of tunicamycin (Sigma) for 16 h.

Protein interactions were then analyzed in the whole cell lysates by IP using anti-FLAG M2 beads, as described above.

Wnt-signaling assay

HEK293 cell lines stably expressing Dkk3 or vector plasmid were co-transfected with an equal ratio of TOP-FLASH luciferase reporter plasmid, a LacZ plasmid, and a third variable plasmid of either V5-Krm1, V5-Krm2, or GFP, following our previously published protocol (Nakamura et al. 2007). Twenty-four hours after transfection, the cells were treated with either control or Wnt3a containing CM for 16 h and then lysates were collected in reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured in a Lumistar Galaxy luminometer (BMG Labtech Inc., Cary, NC, USA) and normalized to β -galactosidase activity. To test the signaling activity of exogenous Dkk3, HEK293 cells were transfected with the Krm and reporter plasmids and the cells were treated with CM containing both Dkk3 and Wnt3a that were mixed in a 1:1 ratio. The control was parental vector CM mixed with Wnt3a-CM. The assays were performed in duplicate in at least four independent experiments. The protein lysates and media were also examined by Western blot analysis to confirm the expression of FLAG-Dkk3, FLAG-Dkk1, V5-Krm1, V5-Krm2, and Wnt3a.

Statistical analysis

Values are reported as mean plus SD. Unpaired-*t*-test was used for statistical analyses.

Results

Dkk3 binds to Krm1 and Krm2

To test the interaction between Dkk3 and Krm proteins, we used GST-pull down and IP. For the GST-pull down assays, truncated forms of Dkk1 and Dkk3 containing partial CRD1 and full CRD2 domains were fused to GST. The CRD2 domain of Dkk1, which is conserved in Dkk3 (Krupnik et al. 1999), has been reported to interact with Krm coreceptors (Davidson et al. 2002; Wang et al. 2008). Equal amounts of GST-Dkk1 and GST-Dkk3 fusion proteins attached to B4 Sepharose beads were incubated with lysate from SH-SY5Y cells transfected with epitope-tagged Krm1 or Krm2. In Figure 1, a 54 kDa band corresponding to Krm1 is present in the GST-pull down product from GST-Dkk1-CRD2 and GST-Dkk3-CRD2 but absent in the GST alone (top panel). A sample of the SH-SY5Y lysates is also shown to the right of the Western blot, and indicates a doublet with lower molecular weight band of 54 kDa, corresponding to the predicted size of Krm1, and a higher molecular band of 75 kDa, corresponding to the glycosylated form of Krm1. A similar outcome was observed using Krm2 as the target protein. A 52 kDa band corresponding to Krm2 was seen in the pull-down products from both GST-Dkk1 and GST-Dkk3 but not in the GST alone (third panel). The lysate lane also shows a 52 kDa band. The second and fourth panels show equal loading of target Krm proteins in the GST-pull-down assays. This experiment demonstrates that both GST-Dkk1 and GST-Dkk3 are associated with the target proteins, Krm1 and Krm2. The GST control did not interact with either Krm1 or Krm2, which indicates the specificity of the interactions between Dkks and Krms.

We were next interested in confirming the interaction between Dkk3 and the Krm proteins in mammalian cells using the full-length proteins. SH-SY5Y cells were transfected with V5-tagged Krm1 or V5-Krm2 and with either the FLAG-Dkk1 or FLAG-Dkk3 constructs. The FLAG-BclXL plasmid was used as a negative control for the co-IP assays. Whole cell lysates were immunoprecipitated with anti-FLAG antibody and the proteins in the immunocomplexes were analyzed by Western blotting. As shown in Figure 2, the target proteins V5-Krm1 and Krm2 are present in the immunoprecipitate from lysates containing Dkk1 or Dkk3, but not from lysates containing BclXL. Dkk1 and Dkk3 interacted with both upper and lower molecular

weight forms of Krm1, presumably the glycosylated and unglycosylated forms of Krm1 (see below). Taken together, these data demonstrate that Dkk3 interacts with Krm1 and Krm2.

Krm1, Krm2, and LRP6 are N-linked glycoproteins, but glycosylation is not critical for the interaction between Dkk3 and Krm proteins

Glycosylation of the Wnt receptors Krm and LRP5/6 has not been reported, although post-translational modifications such as sulfenylation, palmitoylation, and glycosylation have been demonstrated for Wnt ligands and Dkk3 (Krupnik et al. 1999; Komekado et al. 2007). To determine whether Dkk3-interacting proteins are N-linked glycosylated proteins, we performed PNGF digestion. PNGF is commonly used to reduce the N-linked glycomoiety from proteins. SH-SY5Y cells were transfected with V5-tagged Krm1, V5-tagged Krm2, or LRP6, and PNGF digestion was performed on total protein lysates, followed by Western blot detection of the respective proteins. As shown in Figure 3, PNGF treatment decreased the molecular weights of Krm1, Krm2, and LRP6, indicating that they are N-linked glycoproteins. The reduced weight of Krm1 and Krm2 correspond to the predicted weights based on amino acid sequences of 54 kDa and 52 kDa, respectively. The reduced molecular weight of LRP6 was detected around 180 kDa, which corresponds to the predicted molecular weight based on amino acid sequence.

To test whether glycosylation of Dkk3 and Krm receptors is necessary for their interaction, SH-SY5Y co-expressing Dkk3 and Krm were treated with tunicamycin, an N-acetylglucosamine transferase inhibitor that prevents formation of N-acetylglucosamine lipid intermediates and glycosylation of newly synthesized glycoproteins. To detect whether inhibiting these post-translational modifications eliminated the interaction between Krm proteins and Dkk3, the treated whole cell lysates were then immunoprecipitated using anti-FLAG antibody followed by Western blot analysis using the anti-V5 antibody. Samples treated with tunicamycin showed significant decreases in the molecular weights of Krm1, Krm2, and Dkk3, indicating that the tunicamycin treatment was successful (Figure 4). The molecular weights of Krm1 and Krm2 treated with tunicamycin show a reduction in size similar to the PNGF digestion to approximately 54 kDa and 52 kDa, respectively, providing additional evidence that Krm1 and Krm2 are N-linked glycoproteins. However, inhibition of glycosylation by tunicamycin did not interfere with formation of the Dkk3 and Krm1 or Krm2 protein complexes. This result indicates that the glycosylation state of Dkk3 or Krm proteins does not affect their interaction (Figure 4).

Dkk3 and Krm interaction occurs in membrane vesicles

SH-SY5Y cells co-transfected with Dkk3 and Krm were divided into two groups, one half for a whole cell analysis and the other half for subcellular fractionation. As shown in Figure 5(A), Krm1 and Krm2 are mainly detected in the membrane fraction, are absent from the cytoplasmic fraction, and are weakly detectable in the nuclear fraction. The significance of nuclear Krm proteins is currently unclear. In contrast, Dkk3 was primarily localized in the membrane, but there was also weak expression in the cytoplasm. The cytoplasmic expression of Dkk3 was not surprising in spite of it being a glycoprotein because Lee et al. (2009) recently reported that Dkk3 interacts with a cytoplasmic protein β -transducin repeat containing protein in a cervical cancer cell line. Western blot analysis of subcellular marker proteins indicates the purity of each fraction (Figure 5(B)).

To determine the subcellular location of Dkk3 and Krm protein interactions, each fraction was subjected to anti-FLAG co-IP followed by Western blotting with the anti-V5 antibody. Dkk3, Krm1, and Krm2 co-immunoprecipitated only from the membrane fractions and not from the cytoplasmic or nuclear fractions (Figure 5(C)), indicating that Dkk3 interacts with Krm1 and Krm2 proteins in the membrane.

Confocal analysis demonstrated that Dkk3 and Krm1 are located in a perinuclear pattern

To better understand the spatial distribution of Dkk3 and Krm proteins, HEK293 cells were transfected with FLAG-Dkk3 and V5-Krm1 expression constructs followed by immunodetection of Dkk3 and Krm1 and analysis by confocal microscopy. HEK293 cells were used because their flat morphology is more suited for microscopy than SH-SY5Y cells. Krm1 was confined to a perinuclear distribution, while Dkk3 was located throughout the cytoplasm (Figure 6(A)). The pattern of Dkk3 immunodetection was consistent with the subcellular fractionation observed in Figure 5. Colocalization and orthogonal views of Dkk3 and Krm1 are shown in the lower right panel of Figure 6(A), and indicate that there is a substantial overlap between Dkk3 and Krm1 proteins (yellow). Subsequent densitometric analysis of the fluorescent signal of each protein showed overlapping signals in the perinuclear region. Interestingly, not all Dkk3 and Krm1 were co-localized, which likely reflects the different subcellular localization profile observed by Western blotting in Figure 5. Based on the results from Figures 5 and 6, we conclude that Dkk3 and Krm proteins interact in the membrane, most likely in the perinuclear region.

Krm2 diminished Dkk3 potentiation of Wnt signaling

To investigate whether Dkk3 binding to Krm proteins has a functional effect on Wnt signaling, we performed TOP-FLASH luciferase assays in cells co-expressing Dkk3 and Krm1 or Krm2. HEK293 cells stably expressing either Dkk3 or empty vector were used because these cells show Dkk3-mediated potentiation of Wnt3a signaling (Nakamura et al. 2007).

In the green fluorescent protein (GFP) control groups, the addition of Wnt3a significantly increased Wnt signaling in both vector control and Dkk3 stable HEK293 cell lines, as expected (Nakamura et al. 2007). Wnt3a treatment increased luciferase activity by 5.5-fold in the vector control cells (compare the first and third columns, $p < 0.001$) and 7.9-fold in the Dkk3 expressing cells (compare the second and fourth columns, $p < 0.001$) (Figure 7(A)). Furthermore, Dkk3 potentiated Wnt3a signaling by 2.6-fold compared with the vector control (compare the third and fourth columns, $p < 0.01$), which is consistent with our previous findings (Nakamura et al. 2007). When Krm1 was expressed instead of GFP, there was a significant induction of Wnt signaling by Wnt3a in the vector control and Dkk3 cells (Control: compare the fifth and seventh columns, $p < 0.01$, Dkk3: compare sixth and eighth columns, $p < 0.05$). However, when Krm1 was present, there was no potentiation of Wnt signaling by Dkk3 in the presence of Wnt3a (no significant difference between the seventh and eighth columns).

When Krm2 was expressed, Wnt3a was able to induce Wnt signaling (Control: compare the ninth and eleventh columns, $p < 0.01$, Dkk3: compare the tenth and twelfth columns, $p < 0.01$). Dkk3 was also able to potentiate Wnt signaling in the presence of Krm2 (compare the eleventh and twelfth columns, $p < 0.01$), in contrast to Krm1. The amount of the Dkk3-induced response decreased by 43% in the presence of Krm2 compared with GFP (compare the fourth and twelfth columns, $p < 0.01$). These data suggest that Krm2, independently or by interacting with Dkk3, inhibits Dkk3 potentiation of Wnt signaling. Equivalent expression levels of Dkk3, Krm1, and Krm2 between the Wnt3a treated and untreated samples were confirmed by Western blotting (data not shown).

We next explored whether exogenous Dkk3 in the media potentiates Wnt3a to the same extent as when the Dkk3 plasmid is transfected into the cells. Cells expressing Krm receptors were treated with Dkk3 CM that also contained Wnt3a, and compared with cells treated with control CM that contained Wnt3a. Interestingly, the typical Dkk3-induced potentiation of Wnt signaling was not observed in this experiment (Figure 7(B), compare the first and second columns). Similar to the experiment above, Krm2 showed a significant decrease in Wnt signaling compared with the GFP control (compare the first and fifth columns and second and

sixth columns, $p < 0.001$). Krm1 also suppressed Wnt signaling, compared with GFP control (compare the first and third columns, $p < 0.05$). Western blot analysis verified the expression of Krm1 and Krm2 in the cell lysates and Wnt3a and Dkk3 in the CM (Figure 7(C)). Therefore, Dkk3 potentiation of Wnt signaling is only mediated by an intracellular form of the protein.

Dkk3 does not interact with LRP6

LRP5/6 receptors are another class of candidate proteins that may interact with Dkk3. Dkk1 and Dkk2 have previously been shown to interact with LRP5 and LRP6, which are the critical receptors in activating Dkk, Krm, and LRP trimer complexes by endocytosis. This endocytotic process leads to reduced cell surface expression of LRP receptors and diminished Wnt signaling (Mao et al. 2002; Yamamoto et al. 2008). There are numerous reports that indicate that the expression and stability of LRP surface receptors are necessary and sufficient to activate the Wnt pathway (Ding et al. 2008; Kim et al. 2008; Yamamoto et al. 2008). Although LRP5 and LRP6 are believed to play a similar role in regulating Wnt signaling, the relationship between Dkks and LRP6 has been reported more than LRP5 (Mao et al. 2001; Mao and Niehrs 2003; Yamamoto et al. 2008). Therefore, we tested whether Dkk3 regulates LRP6 stability, as a potential mechanism of Dkk3 potentiation of Wnt signaling.

As shown in Figure 8, there was no change in LRP6 levels in HEK293 cells expressing Dkk3 compared with vector plasmids, suggesting that Dkk3 does not modulate LRP6 expression or stability. To test whether Wnt3a stimulation affects the interaction between Dkk3 and LRP6, co-transfected SH-SY5Y cells were incubated with Wnt3a-containing CM, followed by IP with anti-FLAG beads. As shown in Figure 9, Dkk3 did not co-precipitate with LRP6, while Dkk1 did show an interaction. A longer exposure of the film revealed weak LRP6 signal in the sample co-immunoprecipitated with Dkk3. However, it is not possible to distinguish whether this is a specific interaction with Dkk3 or the interaction is coming from endogenous Krm, because Krm proteins interact with LRP6 (Hassler et al. 2007). Dkk1 and LRP6 formed a complex in the lysate, but the secreted forms of Dkk1 and LRP6 did not show such interaction (Figure 9, right panel). Furthermore, Wnt3a treatment did not modulate binding between Dkk3 and LRP6. These data suggest that the ability of Dkk3 to potentiate Wnt3a-mediated signaling is independent of a direct interaction with LRP6.

Discussion

In this study, we examined the molecular interactions between Dkk3 and the Wnt receptors Krm1, Krm2 and LRP6, and tested the functional significance of their interaction in regulating Wnt signaling. We demonstrated the novel findings that Krm1 and Krm2, but not LRP6, interact with Dkk3. Furthermore, we demonstrated that Dkk3 and Krm proteins are not required to be fully matured with glycosylation to interact with each other, and that the interaction takes place in the membrane fraction of the cell. The Dkk3 interaction with Krm1 and Krm2 eliminated or reduced Dkk3 potentiation of Wnt3a-mediated signaling. Lastly, Dkk3 did not modulate LRP6 expression or form protein complexes with LRP6 regardless of the presence of Wnt3a. Therefore, we conclude that Krm1 and Krm2, but not LRP6, are the novel interacting proteins of Dkk3.

Based on our findings from the subcellular fractionation, de-glycosylation, and confocal immunolocalization analysis, we predict that Dkk3 and Krm are interacting in the membrane portion that is located in perinuclear region of the cell, possibly in the endoplasmic reticulum (ER) or Golgi apparatus. Therefore, the interaction between Dkk3 and Krm is not like the prototypic ligand-receptor association found on the membrane surface. This idea is supported by Mao's observation that recombinant Dkk3 failed to bind to surface-expressed Krm receptors in HEK293, or compete against other Dkks binding with Krm (Mao et al. 2002). Also, Cruciat et al. (2006) found that Krm2 lacking transmembrane and cytoplasmic regions does not interact

with Dkk3. These observations further suggest that the interaction between Dkk3 and Krm is taking place within the cell or during post-translational modification, prior to surface expression and secretion of Krm and Dkk3.

The role of LRP6 in Wnt pathway activity has been well characterized. LRP6 is stabilized or phosphorylated by several co-factors, such as R-spondin and caprin-2 that directly bind to LRP6 (Binnerts et al. 2007; Zhang et al. 2007; Ding et al. 2008). Our study showed that Dkk3 does not interact with LRP6 or modulate its expression, which suggests that Dkk3 has a novel mechanism of enhancing Wnt signaling compared with other factors.

We propose the following model based on our findings (Figure 10). Generally, Krm proteins are inhibitors of the Wnt pathway and LRP5/6 are activators (Figure 10(A)) (Mao et al. 2001; Mao and Niehrs 2003). The binding of Dkk3 and Krm in the membrane may lead to sequestering of the Dkk3 and Krm protein complex in intracellular membranes, such as in the ER and Golgi, resulting in reduced surface expression of Krm receptors. A second possibility is that Dkk3 and Krm complexes are co-expressed on the membrane surface and Dkk3 physically blocks the ability of Krm proteins to inhibit the Wnt pathway. Both possibilities would lead to inefficient Krm activity and create a favorable environment for Wnt pathway activation (Figure 10(B)). This idea is supported by the luciferase assay from Figure 7 which demonstrated that Dkk3 significantly increased Wnt signaling compared with vector control in the presence of Krm2. Additionally, secreted Dkk1 is known to inhibit Wnt signaling by binding to Krm and LRP receptors to induce endocytosis (Mao and Niehrs 2003), but binding to the Krm receptor is not required for Dkk1 to inhibit Wnt signaling (Binnerts et al. 2007). Therefore, Wnt signaling is still inhibited when both Dkk1 and Dkk3 are present because Dkk1 and LRP are internalized, which leaves Wnt ligands without their receptor (Figure 10(C)).

In conclusion, our research demonstrates that Krm receptors are unique binding partners of Dkk3, but LRP6 is not. In contrast, Dkk1 binds to Krm and LRP to inhibit the Wnt pathway. The different binding partner profile between Dkk3 and Dkk1 may explain why Dkk3 potentiates Wnt3a-mediated signaling whereas Dkk1 inhibits it.

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Figure 1.

GST-pull down assays demonstrated that Dkk3 interacts with the Krm1 and Krm2 Wnt receptors. GST-pull down assay was performed by incubating GST-Dkk1 and GST-Dkk3 with cell lysates containing V5-Krm1 or V5-Krm2 proteins. Protein complexes bound to GST-fusion proteins were analyzed by immunoblotting (IB) using the anti-V5 antibody. The IB detected Krm1 and Krm2 only in the GST-Dkk1 and GST-Dkk3 complexes and not in the GST complex (first and third panels). Only the lower form of Krm1 and Krm2 interacted, presumably corresponding to the unglycosylated forms. The exposure time of the GST-pull down and lysate IB is 5 s and overnight, respectively. The second and fourth panels demonstrate equal amounts of V5-Krm1 and V5-Krm2 proteins in the lysates input ($N = 5$).

Figure 2.

Dkk3 interacts with both glycosylated and unglycosylated forms of Krm1 and Krm2 Wnt receptors in a mammalian cell line. Anti-FLAG IP followed by anti-V5 IB demonstrated that both V5-Krm1 (A) and V5-Krm2 (B) form protein complexes with FLAG-Dkk1 and FLAG-Dkk3, but not FLAG-BclXI. Furthermore, Dkk1 and Dkk3 were able to form complexes with both glycosylated and unglycosylated forms of Krm1 and Krm2. Expression of FLAG-tagged BclXI, Dkk1, and Dkk3 and V5-tagged Krm in each lysate is shown in the middle panels. The anti-FLAG IP followed by the anti-FLAG IB is used as a positive control (lower panels) ($N = 6$).

Figure 3.

The Wnt co-receptors Krm1, Krm2, and LRP6 are N-linked glycoproteins. SH-SY5Y cells were transfected with Krm1, Krm2, and LRP6 expression constructs. Half of the lysate was subjected to PNGF(+) and the other half was left untreated as the control(-). There was a reduction in the molecular weight for all three proteins, indicating that the secreted Wnt proteins and their receptors are N-linked glycoproteins ($N = 2$).

Figure 4.

Inhibiting glycosylation of Dkk3 and Krm did not eliminate Dkk3 and Krm complex formation. Cells transfected with Dkk3 and Krm expression vectors were either left untreated(-) or treated with 1 μ g/ml tunicamycin(+) overnight to inhibit glycosylation of newly synthesized proteins. Whole cell lysates were subjected to co-IP with anti-FLAG antibody followed by Western blotting for V5-tagged Krm1 and Krm2. The decrease in molecular weight after tunicamycin treatment showed that glycosylation is inhibited, yet the Dkk3-Krm complexes are still co-immunoprecipitated. This result suggests that protein glycosylation is not involved in Dkk3-Krm interactions ($N = 2$).

Figure 5.

Differential localization of Dkk3 and Krms and their interaction in the membrane fraction. (A) The whole cell lysate (W), cytoplasmic (C), membrane (M), and nuclear (N) fractions of transfected SH-SY5Y cells were subjected to Western blot analysis. Anti-V5 and anti-FLAG antibodies were used to determine subcellular localizations of Krm1, Krm2, and Dkk3. The highest intensity bands for Krm1 and Krm2 were found in the membrane fraction and the longer exposure showed a small amount in the nucleus. Dkk3 is mainly in the membrane fraction with weak detection in the cytoplasmic fraction. (B) LRP6 and HSP60 were used as markers for the membrane fraction and Lamin A/C was used for the nuclear fraction to show the purity of the

subcellular fractionation procedure. (C) IP with anti-FLAG antibody showed that Dkk3/Krm interaction takes place in the membrane fraction ($N = 3$).

Figure 6.

Dkk3 and Krm1 colocalize in a perinuclear pattern. (A) HEK293 cells were co-transfected with FLAG-Dkk3 and V5-Krm1 expression vectors followed by immunodetection using antibodies recognizing FLAG-Dkk3 (red) or V5-Krm1 (green) epitopes. 4',6-diamidino-2-phenylindole (DAPI) (blue) was used to stain the nucleus. The spatial localization of Dkk3 and Krm1 was analyzed by establishing region of interest (ROI) 1 (B) and (ROI) 2 (C). The peaks in the densitometric analysis correspond to the relative protein expression. There is a clear overlap between Dkk3 (red) and Krm1 (green) but not with DAPI nuclear staining (blue). Therefore, Dkk3 and Krm1 protein interact most likely in the perinuclear region of the cell, suggesting interaction in the ER ($N = 3$).

Figure 7.

Krm2 abolished Dkk3 potentiation of Wnt3a-mediated signaling. (A) HEK293 cells stably expressing Dkk3 (black bars) or vector control (white bars) were transfected with GFP, Krm1, or Krm2 expression plasmids and the cells were treated with Wnt3a(+) or control(-) CM overnight. Luciferase activity (luciferase/ β -galactosidase) was used to quantify Wnt signaling. Wnt3a significantly increased luciferase activity in all transfections compared with the control CM. The GFP control expressing Dkk3 showed potentiation of Wnt3a signaling ($p < 0.01$), which is consistent with the results previously reported (Nakamura et al, 2007). Dkk3 did not potentiate Wnt signaling in the presence of Krm1, compared with the GFP control, and Krm2 significantly reduced Dkk3 potentiation of Wnt signaling. These data suggest functional interactions among Krm2, Dkk3, and Wnt3a in regulating Wnt signaling. (B) HEK293 cells expressing GFP, Krm1, or Krm2 were treated with Dkk3 CM combined with Wnt3a, or control CM with Wnt3a. Luciferase assays demonstrated that there was no potentiation of Wnt signaling when Dkk3 is present in the media (compare each pair of columns), in contrast to the potentiation when Dkk3 is intracellular (panel A). Krm2 significantly reduced Wnt3a signaling in both treatments. (C) Western blot analysis demonstrating expression of Krm1 and Krm2 in the cell lysates and Wnt3a and FLAG-Dkk3 in the media. p -values: * < 0.05 , ** < 0.01 , *** < 0.001 ($N = 6$ for A and $N = 4$ for B).

Figure 8.

Overexpression of Dkk3 did not modulate LRP6 protein levels. HEK293 stably transfected with Dkk3 or the vector control plasmids were cultured until confluent. Cells were harvested and subjected to Western blot analysis to detect endogenous LRP6 expression. There was no significant difference in LRP6 expression when Dkk3 was overexpressed ($N = 3$).

Figure 9.

Dkk3 does not interact with LRP6. SH-SY5Y cells were transfected with either GFP, FLAG-Dkk1, or FLAG-Dkk3 and the LRP6 expression vector. Cells were treated with either Wnt3a-containing media or control media 48 h after transfection. Cells and media were harvested and immunoprecipitated with anti-FLAG M2 beads, and LRP6 expression was evaluated by Western blotting. LRP6 co-immunoprecipitated with Dkk1 but not with GFP or Dkk3 in the lysates. Dkk1-LRP6 interaction was not seen in media ($N = 2$).

Figure 10.

Proposed model of Dkk3 and Krm in regulating Wnt signaling. (A) Expression of Krm has been reported to inhibit Wnt signaling, presumably through endocytosis of Dkk1, Krm, and LRP receptors (Mao and Niehrs 2003). (B) Our data suggest that Dkk3 forms complexes with Krm proteins in the perinuclear membrane portion of the cell, possibly preventing their surface expression. The reduced surface expression of Krm provides a favorable environment for Wnt ligands to activate Wnt signaling. The ability of Dkk3 to positively modulate the Wnt pathway (Nakamura et al. 2007) does not seem to involve direct interactions or regulation of LRP6 receptors. (C) Unlike Dkk3, Dkk1 binds to the LRP6 receptor and induces endocytosis (Mao

et al. 2002). The reduced surface expression of LRP results in inhibition of Wnt. Krm and Dkk1 are Wnt inhibitors (labeled in red). LRP, Fzd, and Dkk3 are Wnt activators (labeled in green).

Table I

Antibodies used in the study.

Antibody	Dilution	Source
FLAG	1:500 (IHC), 1:1000 (W)	Sigma
V5	10 µg/ml (IHC), 2.5 µg/ml (W)	Sigma
LRP	1:1000 (W)	Cell Signaling
Lamin A/C	1:1000 (W)	Cell Signaling
HSP60	1:1000 (W)	Santa Cruz
Wnt3a	1 µg/ml (W)	Abcam

IHC, immunohistochemistry; W, Western blotting.