LRP6 is internalized by Dkk1 to suppress its phosphorylation in the lipid raft and is recycled for reuse

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Summary

β-catenin-mediated Wnt signaling is crucial in animal development and tumor progression. The phosphorylation of low-density lipoprotein receptor-related protein 6 (LRP6), a single-span transmembrane Wnt receptor, plays a vital role in this signaling. Dickkopf1 (Dkk1) has been shown to inhibit the Wnt–β-catenin pathway, but the mechanism is not yet clear. Here, evidence is presented that Wnt3a-dependent phosphorylation of LRP6 occurs in the lipid raft and that Dkk1 inhibits the formation of a complex between LRP6 and casein kinase 1γ (CK1γ) by removing LRP6 from the lipid raft. Dkk1 internalized LRP6 in a Rab5-dependent mechanism to prevent phosphorylation mediated by CK1γ. The internalized LRP6 was recycled back in a Rab11-dependent mechanism to the cell-surface membrane, and the recycled LRP6 again responded to Wnt3a and Dkk1. Internalized Dkk1 was trafficked in a Rab7-mediated route and degraded in the lysosome. These results suggest that Dkk1 induces the internalization of LRP6 to suppress its phosphorylation in the lipid raft and allows subsequent recycling of LRP6 so that it can be reused for signaling.

Key words: Dkk1, LRP6, Internalization, Recycling, Rab5

Introduction

Wnt proteins constitute a large family of cysteine-rich secreted ligands that control animal development (Logan and Nusse, 2004). There are 19 Wnt members, which exhibit unique expression patterns and distinct functions in development in humans and mice. Wnts control various cellular functions, including proliferation, differentiation, apoptosis, survival, migration and polarity, by regulating multiple intracellular signaling cascades. The interaction of Wnts with their receptors on the cell surface is the first step in transducing an extracellular signal into intracellular responses (Cong et al., 2004). Ten Frizzled (Fz) proteins, which are members of a family of seven-pass transmembrane receptors, have been identified as Wnt receptors (Wang et al., 2006). In addition to Fzs, single-pass transmembrane proteins, such as low-density lipoprotein receptor-related protein 5 (LRP5), LRP6, Ror1, Ror2 and Ryk, have been shown to act as Wnt receptors (Fukuda et al., 2008; Gordon and Nusse, 2006; Green et al., 2008; Kikuchi et al., 2009).

The intracellular signaling pathway activated by Wnts was originally identified as a β-catenin-dependent signaling pathway that is highly conserved among various species (He et al., 2004; Kikuchi et al., 2009). In this pathway, axin functions as a scaffold protein to degrade β-catenin in the absence of Wnt. The binding of Wnts, such as Wnt1 and Wnt3a, to a cell-surface receptor complex consisting of Fz and LRP6 stabilizes β-catenin, and the accumulated β-catenin is translocated into the nucleus, where it stimulates the transcription of target genes (He et al., 2004). Wnt3a promotes rapid phosphorylation of LRP6 by glycogen synthase kinase-3 (GSK-3) and casein kinase 1γ (CK1γ), and this phosphorylation enhances the binding of LRP6 and the axin complex (Bilic et al., 2007; Davidson et al., 2005; Tamai et al., 2004; Zeng et al., 2008; Zeng et al., 2005). The binding between the phosphorylated LRP6 intracellular domain and axin suppresses the function of axin, resulting in the stabilization of β-catenin partly through the caveolin-dependent internalization of LRP6 (Mi et al., 2006; Yamamoto et al., 2006).

The Dickkopf (Dkk) protein family, which comprises four members (Dkk1 to Dkk4), binds to LRP6 and antagonizes the β-catenin pathway (Niehrs, 2006). Dkks contain two characteristic cysteine-rich domains (CRD-1 and CRD-2) separated by a linker region of variable length (Glinka et al., 1998). Two possible mechanisms by which Dkk1 inhibits the β-catenin pathway have been proposed. One is that Dkk1 prevents the formation of a ternary complex of Wnt, Fz and LRP6 on the cell surface by binding to LRP6 (Bafico et al., 2001). In this mechanism, Dkk1 does not induce the internalization of LRP6 (Semënov et al., 2008). Another possibility is that Dkk1 induces the internalization of LRP6 through a clathrin-mediated route, thus removing LRP6 from the plasma membrane (Mao et al., 2002; Yamamoto et al., 2008). Thereafter, whether Dkk1 induces the internalization of LRP6 is not clear and the fates of internalized Dkk1 and LRP6 are not well understood. In this study, it was found that Dkk1 induces the internalization of LRP6 in a Rab5-dependent mechanism to prevent the Wnt3a-dependent phosphorylation of LRP6 in the lipid raft. In addition, it was shown that the internalized LRP6 is recycled in Rab11-dependent and actin-filament-dependent mechanisms to be reused, and that Dkk1 is transported to the degradation pathway in a Rab7-dependent mechanism.

Results

Dkk1 inhibits Wnt3a-dependent phosphorylation of LRP6 in the lipid raft fraction

It was reported previously that overexpressed LRP6 is present in both the lipid raft and non-raft fractions (Yamamoto et al., 2006). To examine the distribution of endogenous LRP6, wild-type
HEK293T cells were fractionated in the presence of Triton X-100 to separate them into detergent-resistant microdomains (DRMs) and non-DRMs. Although DRMs do not necessarily correspond to the lipid raft in cells, detergent insolubility is a valuable and widely used method to identify proteins that are localized to the lipid raft and probably function there. Endogenous LRP6 was observed in both DRMs and non-DRMs, and Wnt3a did not affect the distribution of LRP6 between the two fractions (Fig. 1A). Although Wnt3a bound to LRP6 in both fractions with a similar efficiency (Fig. 1B), Wnt3a induced the phosphorylation of LRP6 at Ser1490 and Thr1479, which are known to be phosphorylated by GSK-3β and CK1γ, respectively, only in DRMs (Fig. 1C). It has been shown that caveolin plays a role in the Wnt3a-dependent internalization of LRP6 and accumulation of β-catenin (Yamamoto et al., 2006; Yamamoto et al., 2008). Knockdown of caveolin1 affected neither the distribution of LRP6 between DRMs and non-DRMs nor the Wnt3a-dependent phosphorylation of LRP6, but it inhibited the accumulation of β-catenin (Fig. 1D,E). Nystatin, which binds to cholesterol and disrupts the lipid raft, inhibited Wnt3a-dependent LRP6 phosphorylation and β-catenin accumulation (Fig. 1E). These results suggested that Wnt3a induces the phosphorylation of LRP6 in the lipid raft, but that this phosphorylation is not sufficient for the stabilization of β-catenin. Moreover, these findings also indicated that the presence of lipid rafts is important for Wnt3a signaling.

Dvl and CK1 are important components in the Wnt-β-catenin pathway (Knippschild et al., 2005; Wharton, 2003). Endogenous Dvl was distributed mainly into non-DRMs and partly into DRMs (Fig. 2A). Wnt3a induced a mobility shift of Dvl in an SDS-polyacrylamide gel in both microdomains (Fig. 2A). The upper band of Dvl was a phosphorylated form because it disappeared following treatment with alkaline phosphatase (data not shown). It was notable that endogenous Dvl showed a mobility shift without Wnt3a stimulation in non-DRMs, especially fractions 9 and 10, and that Wnt3a increased the ratio of the upper band (Fig. 2A). CK1γ was present in DRMs, whereas CK1α and CK1ε were present in non-DRMs (Fig. 2B), and their distribution was consistent with the findings that CK1γ and CK1ε phosphorylate LRP6 and Dvl, respectively (Davidson et al., 2005; Kishida et al., 2001).

Dkk1 did not affect the distribution of CK1γ, Dvl or caveolin1 in DRMs or non-DRMs (Fig. 2C). However, Dkk1 removed LRP6 from DRMs at the endogenous level (Fig. 2D), consistent with the overexpression of LRP6 (Yamamoto et al., 2008). Formation of a complex between LRP6 and CK1γ or caveolin1 was enhanced by Wnt3a and inhibited by Dkk1 (Fig. 2E). CK1γ formed a complex with caveolin1 irrespective of Wnt3a stimulation, and Dkk1 inhibited their association (Fig. 2E). Consistent with these results, Dkk1 reduced the amount of Wnt3a-induced phosphorylation of LRP6 at Ser1490 and Thr1479 in DRMs (Fig. 2F). These findings suggested that Dkk1 suppresses the phosphorylation of LRP6 at Thr1479 by inhibiting the association of CK1γ with LRP6 and by reducing the amount of LRP6 in the lipid raft. Furthermore, inhibition of the interaction between CK1γ and LRP6 by Dkk1 might affect the phosphorylation of LRP6 at Ser1490.

**LRP6 is internalized by Dkk1 and recycled to the cell-surface membrane**

It was shown previously that Dkk1 induces the internalization of LRP6 and inhibits the β-catenin pathway, but the results were obtained from experiments using the overexpression of LRP6 (Mao et al., 2002; Yamamoto et al., 2008). Therefore, removal of endogenous LRP6 from the cell-surface membrane was investigated by two different methods. First, cell-surface proteins were labeled with the impermeant, non-cleavable biotin analog sulfo-NHS-LC-biotin at various time points after Dkk1 stimulation. Biotinylated proteins were immunoprecipitated with anti-LRP6 antibody and then the precipitates were probed with streptavidin–horse radish peroxidase (HRP). Dkk1 reduced the levels of cell-surface LRP6 in both time- (up to 60 minutes) and dose-dependent manners in HeLaS3 cells (Fig. 3A-C). The dose-dependent effect of Dkk1 on the internalization of LRP6 was similar to its effect on the inhibition of Wnt3a-dependent LRP6 phosphorylation (Fig. 3B,C). Similar results were also observed in HEK293 cells with endogenous LRP6 and overexpressed LRP6 (Fig. 3D and supplementary material Fig. 361).
due to protein synthesis because LR6P was recycled in cells treated with cycloheximide to block protein synthesis. In addition, these findings were confirmed by two biotinylation methods. The first method, using sulfo-NHS-SS-biotin, showed that cell-surface LR6P is reduced at 30 minutes after Dkk1 stimulation and LR6P was recycled back to the cell surface in a time-dependent manner after the removal of Dkk1 (Fig. 4B). The recycling of endogenous LR6P was also monitored using sulfo-NHS-SS-biotin. LR6P was indeed internalized within 30 minutes by the stimulation with Dkk1, and LR6P was recycled back to the cell surface at 90 minutes after the removal of Dkk1 (Fig. 4C). LR6P-GFP recycled to the cell-surface membrane was internalized again when the cells were stimulated with Dkk1 and LR6P was recycled back to the cell surface at 90 minutes after the removal of Dkk1 (Fig. 4E).

Although Dkk1 was colocalized with LR6P-GFP as small puncta at 10 and 60 minutes (Fig. 4A), it was difficult to detect Dkk1 at 120 and 180 minutes. However, Dkk1 could be detected as cytoplasmic puncta in the presence of lysosomal inhibitors such as chloroquine or bafilomycin A1 (Fig. 4F), suggesting that Dkk1 is degraded after it dissociates from LR6P. Thus, LR6P-GFP also recycled to the cell-surface membrane and Dkk1 is degraded, probably in the lysosome.

The trafficking of LR6P is regulated by Rab small GTPases
Rab small GTPases exert regulatory functions in exocytotic and endocytic transport, through the recruitment of specific effector proteins to the membrane to which they are localized (Sonnichsen
et al., 2000; Zerial and McBride, 2001). Rab5, through its effector proteins, including early endosome antigen 1 (EEA1) and Rabenosyn-5, organizes a membrane domain that defines the site of entry into early endosomes. At 30 minutes after stimulation with Dkk1, internalized LRP6-GFP was colocalized with EEA1 and Rab5, and the internalized Dkk1-FLAG was also colocalized with Rab5 (Fig. 5A). The expression of a dominant-negative form of Rab5 (HA-Rab5S34N) suppressed the internalization of LRP6-GFP (Fig. 5B), indicating that LRP6 internalized by Dkk1 was transported to early endosomes by the actions of Rab5 (Fig. 5B). Rab11 plays a role in the recycling of internalized proteins through recycling endosomes, and Rab7 is suggested to have a role in late endocytic trafficking, including the late endosome to lysosome pathway (Chavrier et al., 1990; Feng et al., 1995; Sonnichsen et al., 2000). Although LRP6-GFP was colocalized with HA-Rab11 at 90 minutes after stimulation with Dkk1, it did not colocalize with HA-Rab7 (Fig. 5C). LRP6 and Rab11 were observed as having highly punctuate and scattered patterns, suggesting that they were in recycling endosomes, because recycling endosomes are clustered in the perinuclear region (Marsh et al., 1995). A dominant-negative form of Rab11 (HA-Rab11S25N) did not affect the Dkk1-dependent internalization of LRP6-GFP but suppressed the reappearance of LRP6-GFP at the cell-surface membranes and retained it in the perinuclear region at 120 minutes after stimulation with Dkk1 (Fig. 5D). Consistent with these results, the Wnt3a-dependent phosphorylation of LRP6 was decreased significantly at 120 and 150 minutes after treatment with Dkk1 when Rab11S25N was expressed (Fig. 5E). Although LRP6-GFP was retained in the cytoplasm in cells expressing Rab11S25N, Dkk1-FLAG was not detected at 120 minutes after Dkk1 treatment, suggesting that Dkk1 is not recycled to the plasma membrane with LRP6 (Fig. 5D). The expression of a dominant-negative form of Rab7 (HA-Rab7T22N) did not influence the internalization or recycling of LRP6-GFP (Fig. 5D). However, HA-Rab7T22N induced the accumulation of Dkk1-FLAG, suggesting that Dkk1 is transported to the lysosome in a Rab7-dependent mechanism. Thus, Rab5 is involved in the internalization of the complex of Dkk1 and LRP6. Rab7 and Rab11 are involved in the degradation of Dkk1 and the recycling of LRP6, respectively.

### The actin cytoskeleton is required for the recycling of LRP6

The roles of actin filaments and microtubules in the internalization and recycling of LRP6 were examined. Cytochalasin D, an actin-filament-disrupting reagent, and nocodazole, a microtubule-disrupting reagent, did not inhibit the internalization of LRP6-GFP induced by Dkk1 (Fig. 6A). Cytochalasin D and nocodazole had no influence on the Wnt3a-dependent accumulation of β-catenin, and Dkk1 suppressed Wnt3a-dependent β-catenin stabilization in cells treated with these reagents (Fig. 6B). However, the recycling of LRP6-GFP was suppressed by cytochalasin D but not by nocodazole (Fig. 6A). At 180 minutes after stimulation with Dkk1, the phosphorylation of LRP6 by Wnt3a was not detected in the...
The phosphorylation of LRP6 by GSK-3 and CK1γ is essential for the Wnt-dependent accumulation of β-catenin (Biloc et al., 2007; Davidson et al., 2005; Zeng et al., 2008; Zeng et al., 2005). In this model, Wnt3a induces a Dvl-dependent aggregate of LRP6, which promotes CK1γ-mediated phosphorylation and signalosome formation (Biloc et al., 2007). Dvl associated with the axin complex is recruited to Fz and the phosphorylated LRP6; thereby, GSK-3 bound to axin further enhances the phosphorylation of LRP6 (Yamamoto et al., 2006; Zeng et al., 2008). In addition, caveolin-mediated internalization was shown to be required for Wnt3a-dependent activation of the β-catenin pathway (Yamamoto et al., 2006; Yamamoto et al., 2008). In this study, it was found that the phosphorylation of LRP6 occurs in the lipid raft but not in the non-lipid raft, although LRP6 is present in both fractions. Because Wnt3a bound to LRP6 in both fractions, it is unlikely that the affinity of LRP6 for Wnt3a is different. CK1γ was localized to the lipid raft, whereas CK1δ and CK1ε were in the non-lipid raft. It was demonstrated that CK1γ, CK1δ, and CK1ε phosphorylate LRP6, β-catenin, and Dvl, respectively (Davidson et al., 2005; Hino et al., 2003; Liu et al., 2002). Therefore, the subcellular localization of the CK1 subfamily might determine the substrate specificity of the Wnt signal components. The localization of CK1γ in the lipid raft could be necessary for the Wnt3a-dependent phosphorylation of LRP6.

Wnt3a enhanced the interaction between LRP6 and CK1γ or caveolin, and the formation of a complex between CK1γ and caveolin required LRP6. These results suggest that these proteins form a complex in response to Wnt3a and support the findings that CK1γ is involved in the phosphorylation of LRP6 in the lipid raft. Dklk1 induced clathrin-dependent internalization of endogenous LRP6 by removing LRP6 from the lipid raft to the non-lipid raft and inhibited the Wnt3a-dependent phosphorylation of LRP6. Therefore, it is possible that Dklk1 inhibits the Wnt3a-dependent formation of a complex between LRP6, CK1γ and caveolin by removing LRP6 from the lipid raft, and that, as a consequence, it suppresses the Wnt3a-dependent phosphorylation of LRP6 at Thr1479. In addition,
inhibition of the interaction between CK1γ and LRP6 by Dkk1 might also affect the phosphorylation of LRP6 at Ser1490.

It was found that the Wnt3a-dependent phosphorylation of LRP6 was inhibited by nystatin treatment but not by caveolin1 knockdown, although these manipulations suppressed the internalization of LRP6 and the accumulation of β-catenin. These results suggested that the phosphorylation of LRP6 occurs in the lipid raft without internalization, but that its phosphorylation is not sufficient for the accumulation of β-catenin. Therefore, many cell-surface events, including receptor clustering, the translocation of the axin complex, and the formation and internalization of coated pits, are necessary for the triggering of Wnt3a-dependent β-catenin signaling.

**Trafficking routes of Dkk1 and LRP6**

It was shown previously that Dkk1 induces the internalization of LRP6 in a clathrin-dependent manner (Mao et al., 2002; Yamamoto et al., 2008), and another report demonstrated that Dkk1 does not affect the rate of LRP6 internalization (Semënov et al., 2008). One of the differences between these experiments was in the experimental conditions, in terms of whether overexpressed LRP6 or endogenous LRP6 was used. The present study investigated the removal of endogenous LRP6 from cell-surface membranes using two different cell-surface biotinylation methods. It was found that Dkk1 induces the internalization of endogenous LRP6 in HeLaS3 and HEK293 cells via a clathrin-mediated route. At present, the reasons for the differences in these results are not known.

It was reported originally that Kremen2 acts as a Dkk1 receptor and that Dkk1 induces the removal of overexpressed LRP6 from the cell surface in HEK293T cells that overexpressed both LRP6 and Kremen2 (Mao et al., 2002). The immunocytochemical studies in Fig. 4A were done without Kremen2 overexpression. Overexpression of Kremen2 enhanced the internalization rates of
LRP6 slightly (supplementary material Fig. S2). However, it was shown that Kremen1 and 2 are not universally required for Dkk1 function using knockout mice (Ellwanger et al., 2008). The knockdown of Kremen1 and 2 did not affect the internalization of LRP6 (supplementary material Fig. S3). Moreover, Dkk1 mutants that do not bind to Kremen1 or 2 still antagonized Wnt3a-dependent Lef-1 activity (Wang et al., 2008). Therefore, Kremen might not be an essential mediator for Dkk1 function.

The present study found that Dkk1 and LRP6 colocalize with Rab5 and EEA1, and that a dominant-negative form of Rab5 inhibits the Dkk1-dependent internalization of LRP6. These results suggest that the complex of Dkk1 and LRP6 moves to the early endosomes via a Rab5-dependent process. Rab11 is involved in the regulation of the recycling pathway (Sonnichsen et al., 2000). It was also shown that the internalized LRP6 appears on the cell-surface membrane at 2-3 hours after stimulation with Dkk1 and that a dominant-negative form of Rab11 blocks this process, suggesting that LRP6 can be recycled at least through a Rab11-dependent recycling pathway. Furthermore, recycled LRP6 responded to both Wnt3a and Dkk1 functionally. It was reported previously that recycling endosomes are enriched in raft lipids, including sphingomyelin and cholesterol, and that late endosomes contain low amounts of these lipids (Gagescu et al., 2000). It is intriguing to speculate that the selective incorporation of lipid raft components in recycling endosomes contributes to regulate protein and lipid sorting and trafficking. Internalized LRP6 might be redistributed to the lipid raft and non-raft in the endosomes or after being recycled back to the plasma membrane.

Dkk1 was observed with LRP6 in the early endosome, but afterwards it could not be detected. Because Dkk1 was detected by treatment of the cells with chloroquine and bafilomycin A1, Dkk1 might be degraded in the lysosome. This was supported by the fact that Dkk1 is observed in cells overexpressing a dominant-negative form of Rab7, because Rab7 has been shown to be localized to the late endosome and to be necessary for the lysosomal degradation of ligands (Chavrier et al., 1990). The trafficking pathways of Dkk1 and LRP6 were consistent with the well-established concept that internalized molecules arrive in the early endosome, where uncoupling of ligands and receptors occur, and that ligands destined for degradation, such as LDL, are forwarded to the late endosome and lysosome, whereas receptors such as the LDL receptor are recycled to the cell-surface membrane via the recycling endosome. However, some LRP6 might also be delivered to the lysosome and degraded, because it was reported that the half-life of LRP6 is estimated to be about 5 hours (Semënov et al., 2008).

**Actin and recycling of LRP6**

The cytoskeleton, especially actin filaments, has been implicated in vesicular trafficking, including endocytosis (Qualmann et al., 2000). However, a number of studies both supported and refuted the involvement of actin filaments in receptor-mediated endocytosis: inhibitors of actin polymerization blocked the internalization of cell-surface receptors such as thromboxane A2 receptor and β2-adrenergic receptor (β2AR) (Laroche et al., 2005; Volovyk et al., 2006), whereas these inhibitors do not necessarily affect the internalization of chemokine receptors and β2AR (Fujimoto et al., 2000; Millman et al., 2008; Zaslaver et al., 2001). These conflicting data might depend on the agents that chemically disrupt the actin dynamics or the methods used to evaluate the internalization of receptors. The present study showed that disrupting actin filaments does not affect the internalization of LRP6 in response to Dkk1 but does inhibit its recycling. Moreover, actin filaments were not required in Wnt3a...
action for the accumulation of β-catenin, or in Dkk1 action for the inhibition of Wnt3a action. It was demonstrated previously that the caveolin-mediated internalization of LR6P is required for Wnt3a-dependent β-catenin accumulation (Yamamoto et al., 2006) and that the clathrin-mediated internalization of LR6P plays a role in the Dkk1-dependent inhibition of β-catenin accumulation (Yamamoto et al., 2008). Taken together, these results suggest that actin filaments are not required for Wnt3a-dependent receptor-mediated internalization and signaling, at least in the cells used in this study.

It has been reported that rapid recycling of β2AR is mediated by the cytoskeleton-associated recycling and transport (CART) complex, which consists of Hrs, actin-4, brain-expressed RING finger protein (BERP), and myosin Vb (Millman et al., 2008; Yan et al., 2005). The recycling of LR6P was not affected by a dominant-negative form of Rab4 (data not shown), which has been shown to be involved in the rapid recycling of receptors. Therefore, it is unlikely that actin filaments are involved in the rapid recycling of LR6P. Annexin-A2-dependent polymerization of actin was shown to be required for the transport from early to late endosomes (Morel et al., 2009). Although how actin filaments are involved in the slow recycling process through Rab11 is not clear, it is intriguing to imagine that a certain complex including actin regulates the transport from early to recycling endosomes or from recycling endosome to the plasma membrane.

Receptor-mediated endocytosis and recycling of a receptor are not generally affected by microtubule depolymerization (Matter et al., 1990; McGraw et al., 1993), and the present results also showed that microtubules are not involved in the Dkk1-dependent internalization or recycling of LR6P. However, some reports showed that apical recycling is inhibited in nocodazole-treated epithelial cells (MDCK cells) (Breitfeld et al., 1990). Therefore, it might be possible that microtubules are involved in the trafficking of Dkk1-induced LR6P, depending on cell type.

**Materials and Methods**

**Materials and chemicals**

pCS2-LR6P-EGFP, pcS2/Flag-LR6P, pCS2/Kremen2, pCMV-FLAG/Mesd and anti- phospho-T1479 LR6P (T1479P) antibody were kindly provided by Christof Niehrs (Division of Molecular Embryology, DFKZ, Heidelberg, Germany) (Davidson et al., 2005). Rab5 WT, Rab5 S34N, Rab11 WT, Rab11 S25N, Rab7 WT and Rab7 S22N expression vectors were donated by Takuya Sasaki (Department of Biochemistry, Tokushima University, Tokushima, Japan). Anti-Wnt3a polyclonal and anti-Dvl polyclonal antibodies were prepared as described previously (Kishida et al., 2004). Conditioned media (CM) containing Wnt3a and Dkk1-FLAG were prepared and Dkk1-FLAG was purified from CM as described previously (Bafico et al., 2001; Yamamoto et al., 2006). When the cells were stimulated with Wnt3a or Dkk1 CM, half or all of the culture medium was replaced with CM. Anti-LR6P monoclonal and anti-S1490P polyclonal antibodies were purchased from Cell Signaling Technology (Danvers, CA). Anti-caveolin polyclonal, anti-GSK3β monoclonal, anti-β-catenin monoclonal, anti-clathrin monoclonal, anti-EEA1 monoclonal antibodies were from BD Biosciences (San Jose, CA). Anti-FLAG M2 monoclonal and anti-HA monoclonal (16B12) antibodies were from Sigma-Aldrich (St Louis, MO) and Covance (Princeton, NJ), respectively. Anti-C41t1x antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-transferrin receptor antibody was from Zymed Laboratories (San Francisco, CA). Other materials were from commercial sources.

**Preparation of DRM fractions**

HEK293T or HeLa S3 cells (in three 100-mm diameter dishes) were lysed in 0.5 ml of ice-cold TNE buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl and 5 mM EDTA- NaOH pH 8.5 containing 0.4% Triton X-100, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μM phenylmethylsulfonyl fluoride). Then the cell lysates were further homogenized using a Dounce homogenizer (40 strokes) and subsequent passage through a 25-gauge needle (Brown and Rose, 1992). Lysates (0.5 ml) were mixed with 0.5 ml of 80% (w/v) sucrose in TNE and overlayed with 2 ml of 35% sucrose in TNE, followed by 1 ml of 5% sucrose in TNE. The gradients were centrifuged at 190,000 g for 18 hours at 4°C in an RPS 56/7 rotor (Hitachi, Tokyo, Japan). Fractions (400 μl) were harvested from the top of the gradient. Aliquots were probed with the indicated antibodies.

**siRNAs**

The following RNA duplexes were used: human clathrin (sense), 5'-CCUG-CGCUUGGUGAUCAGTCT'3'; human caveolin 1 (sense), 5'-GAAGGACA-GUGUACAGCTT3' and 5'-CCGUAGUUGAGAUUCAGUGTT3'; randomized control (sense). 5'-CAGUGCCUUUGCAGUGTT3'. Double-stranded RNA oligonucleotides were annealed in vitro before transcription. The two RNA duplexes for caveolin 1 were pooled and used.

**Biotinylation internalization assay using sulfo-NHS-LC-biotin**

HeLa S3 or HEK293 cells were treated with Dkk1 CM, and then the cells were incubated with 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) at 4°C for 30 minutes (Yamamoto et al., 2006). After quenching of excess biotin with 50 mM NH4Cl, the cells were lysed in 0.2 ml of TNE buffer, except that 1% Triton X-100 and 0.4% sodium deoxycholate were used instead of 0.4% Triton X-100. The lysates were immunoprecipitated with anti-LR6P antibody, and the immunoprecipitates were analyzed by immunoblotting with streptavidin-HRP (cell-surface LR6P) or anti-LR6P antibody (total LR6P). Alternatively, the lysates were precipitated using neutravidin-agarose beads, and the precipitates were probed with anti-LR6P antibody.

**Biotinylation recycling assay using cleavable sulfo-NHS-SS-biotin**

After biotinylation with 0.5 mg/ml sulfo-NHS-SS-biotin, cells were stimulated with Dkk1 CM at 37°C for 30 minutes. The remaining biotin on the cell surface was stripped using 0.5 mM MesNa in 100 mM Tris-HCl pH 8.6 (containing 100 mM NaCl and 2.5 mM CaCl2) at 4°C for 30 minutes. Cells were then lysed in 0.2 ml of TNE buffer except that 1% Triton X-100 and 0.4% sodium deoxycholate were used instead of 0.4% Triton X-100. Biotinylated proteins that protected from MesNa were precipitated using neutravidin-agarose beads, and the precipitates were probed with anti-LR6P antibody.

**Internalization of LR6P-GFP**

HEK293 cells were used for internalization assays as described previously (Yamamoto et al., 2006; Yamamoto et al., 2008). The cells were seeded onto 18-mm glass coverslips coated with poly-D-lysine (Sigma, St Louis, MO). pCS2/LR6P-EGFP and pCMV-Myc/Mesd were transfected into the cells using Lipofectamine 2000 (Invitrogen). pCS2/Kremen2 was also transfected in some experiments. At 24–48 hours after transfection, the cells were incubated with ice-cold binding medium (DMEM containing 20 mM HEPES-NaOH pH 7.5 and 0.1% BSA) for 30 minutes and treated with Dkk1 CM for 1 hour at 4°C. After unbound Dkk1 was removed by washing with cold PBS, internalization was initiated by adding warm DMEM at 37°C. For observing the internalization of LR6P-GFP, the cells were washed with cold PBS to stop internalization. The cells were viewed directly using a confocal microscope (LSM510, Carl-Zeiss, Jena, Germany) to observe LR6P-GFP. When necessary, HEK293 cells were pretreated with 50 μg/ml cycloheximide for 2 hours or with 100 μl chloroquine, 100 μm bafilomycin A1, 2 μM cytchalasin D or 20 μM nocodozole for 30 minutes at 37°C before incubation with ice-cold binding medium. To observe Dkk1-FLAG, the cells were treated with Dkk1 CM containing anti-FLAG antibody for 1 hour at 4°C and internalization was initiated as described above. After the cells were fixed and permeablized, they were probed with the second antibody. The images shown are representative of 100 microscopic fields in five independent experiments.

**Quantification of internalized LR6P**

To quantify the internalization of LR6P-GFP, the appearance of the intracellular localization of LR6P was classified into three types with regard to the distribution pattern of LR6P-GFP and the number of puncta in the cytosol. The first type showed clear localization at the cell surface, with a few puncta in the cytosol. The second type showed localization to both the cell surface and puncta in the cytosol. The third type showed the disappearance of the cell-surface distribution, with more than 20 puncta in the cytosol. More than 100 cells were evaluated in each experiment.

**Immunocytochemistry**

Cells grown on glass coverslips were fixed for 15 minutes in PBS containing 4% (w/v) paraformaldehyde and then permeablized with PBS containing 0.2% (w/v) Triton X-100 and 2 mg/ml BSA for 30 minutes. The cells were stained with anti-HA or anti-EEA1 antibody before confocal microscopy was performed. The images shown are representative of 100 microscopic fields of at least three independent experiments.
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