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Multiple PPPS/TP motifs act in a combinatorial fashion to transduce Wnt Signaling through LRP6

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Abstract

Binding of Wnt to Frizzled, and either of two members of the low-density-lipoprotein receptor-related protein family, LRP5/6, leads to β -catenin activation by a poorly understood mechanism. LRP5/6 exhibit five highly conserved PPPS/TP motifs in their intracellular region, among which the first PPPS/TP site is rapidly phosphorylated upon Wnt stimulation. By the use of full-length LRP6 mutants harboring multiple mutations involving the five PPPS/TP motifs, we found that this first PPPS/TP phosphoacceptor site is alone not sufficient or strictly necessary for β -catenin activation. Instead, we show that each LRP6 PPPS/TP motif contributes in a combinatorial fashion to activate the canonical Wnt- β -catenin pathway.

Keywords

Wnt; LRP6; β -catenin; nuclear signaling; cancer

1. Introduction

The Wnt family of secreted glycoproteins plays a key role in developmental patterning and cellular proliferation, and its aberrant activity has been directly linked to many highly prevalent human cancers [1-4]. The intracellular signaling pathways initiated by Wnt have been historically divided into canonical, which result in the nuclear accumulation of the transcription factor β -catenin, and noncanonical, which include $[Ca^{2+}]$ elevation, JNK phosphorylation, Rho activation, and polarity signals [3]. Abnormal hyperactivation of the canonical Wnt pathway, which may result from alterations in the coding sequence of β -catenin, Axin or the tumor suppressor protein adenomatous polyposis coli (APC), is directly implicated in tumorigenesis, including colon cancer and therefore this branch of Wnt signaling has received intense mechanistic scrutiny.

The crux of the canonical mechanism is the nuclear accumulation of β -catenin [5]. In the absence of Wnt stimulation, β -catenin binds the Axin-APC-GSK3- β complex, which results in the constitutive phosphorylation and consequent ubiquitin-dependent degradation of β -

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catenin in the proteasome. Wnt binds to members of the Frizzled family of seven-transmembrane receptors and to LRP5 or LRP6, two members of the low-density-lipoprotein receptor-related (LDL-R) protein family, which inhibits the Axin complex [6-8], thereby promoting the accumulation of unphosphorylated β -catenin [9,10]. β -catenin subsequently translocates to the nucleus where it complexes with TCF/LEF transcription factors, thereby regulating the expression of genes controlling proliferation and cell cycle progression [5]. How the interaction of Wnt, Frizzleds and LRP5/6 ultimately causes the activation of β -catenin is still not fully understood.

LRP6 has five phosphorylatable motifs (PPPS/TP) that are evolutionarily conserved from flies to humans [11]. Among them, the first PPPS/TP motif, including serine 1490, is rapidly phosphorylated upon Wnt activation, and it is believed to be important for Wnt signaling to β -catenin [11]. Glycogen Synthase Kinase 3 β (GSK3 β) and Casein Kinase 1 (CK1) can cooperate to phosphorylate the first PPPS/TP site of LRP6 in response to Wnt [7], even though emerging evidence suggests distinct roles for GSK3 β and CK1 isoforms in Wnt stimulation of β -catenin [7,8,12-15]. Surprisingly, however, we found that in the context of the full length LRP6, phosphorylation of serine 1490 was not sufficient, and only marginally necessary to promote β -catenin activation by Wnt. This prompted us to explore the relative contribution of each of the conserved PPPS/TP motifs. We engineered a full-length LRP6 mutant lacking all five PPPS/TP motifs followed by the reconstitution of each of these five sites in multiple combinations. This systematic add-back approach revealed that no single PPPS/TP phosphoacceptor site is sufficient for LRP6 signaling. Instead, at least four of the five PPPS/TP sites are required for the full activation of β -catenin. Furthermore, our findings suggest that these PPPS/TP motifs are not functionally equivalent but act in a combinatorial fashion to transduce the canonical Wnt- β -catenin pathway.

2. Materials and Methods

2.1. Cell culture, reporter assays, and conditioned medium

HEK-293T cells stably transfected with the SuperTOPflash (STF) reporter plasmid, in which several TCF4-binding repeats drive firefly luciferase expression [16], were transfected in 96 well plates with 100 ng of LRP6 WT, mutant, or GFP, and 20 ng of a polymerase II promoter-driven renilla luciferase construct per well, using Lipofectamine PlusTM reagent (Invitrogen). Cells were grown for 16-24 hours and incubated for an additional 6-10 hours with either Wnt3a conditioned medium or control medium. Luciferase assays were performed using a Dual Glo Luciferase KitTM (Promega) and a VictorTM 3V luminometer (Perkin-Elmer). The firefly luciferase value in each well was normalized to the corresponding renilla luciferase readout. Conditioned medium was prepared using L cells that contain either Wnt3a expression plasmid or an empty vector (referred to as control) (ATCC), as described [17].

2.2. DNA constructs

All mutant LRP6-ICD constructs were engineered from human LRP6-ICD cDNA (NM_002336, nucleotides 4190-4842), subcloned with 5'-BamHI and XbaI-3' into a pGEX-4T3 bacterial expression vector (Invitrogen). Mutagenesis reactions were performed using the Quikchange Mutagenesis KitTM (Stratagene) and mutagenic primers containing the desired serine or threonines to alanine mutation (Invitrogen). For site 2, in which two threonines sit between the flanking prolines (PPTTP), both threonines were mutated in tandem for simplification. For the add-back reactions, the native amino acid was substituted for alanine. Following sequence confirmation, ICD mutants were cloned back into full-length hLRP6 cDNA in the pCDNAIII-V5-6HisA (Invitrogen) mammalian expression vector.

2.3. Immunofluorescence

HEK-293T cells were plated onto poly-D-lysine coated glass coverslips and transfected with ExGen 500 transfection reagent (Fermentas). After 24 hours, cells were fixed with PBS-paraformaldehyde 4% 15 min, permeabilized in PBS-triton 0.5% 5 min and stained with primary and fluorescent conjugated secondary antibodies (Jackson ImmunoResearch), and mounted in DAPI-containing medium (Vectashield, Vector Labs). Axin (Zymed-Invitrogen) and β -catenin (Sigma) staining was visualized using TCS/SP2 Leica microscope (NIDCR Confocal Facility, NIH, Bethesda, MD). Quantification of the number of cells containing Axin clusters was obtained by counting the number of α -V5 immunolabeled positive cells that displayed punctate staining after α -Axin immunolabeling.

2.4. Western blot analysis

Cells transfected as described above were treated with either control or Wnt3A conditioned medium for 45 minutes, and lysed (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1mM Na₃VO₄, 10 ug/mL Aprotinin, 10 ug/mL Leupeptin, 1mM PMSF) on ice. Equal amount of soluble protein for each sample was subjected to SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Millipore Corp.). The membranes were then probed with α -pLRP6^{S1490} (Cell Signaling Technology), which specifically recognizes LRP6 phosphorylated at serine 1490, α -V5 (Invitrogen) or α -tubulin (Santa Cruz Biotechnology) antibodies. Proteins were visualized with Immobilon Western reagent (Millipore Corp.).

2.5. Quantitative RT-PCR analysis

Total RNA was isolated from HEK-293T-STF cells transfected with GFP control, LRP6 wildtype or LRP6 mutant (AAAAA) after treatment with control or Wnt3A conditioned media for 24 h with the RNeasy isolation kit (Qiagen). Three micrograms of total RNA were used for each RT reaction using the Superscript II reagent (Invitrogen) as previously reported [18]. Quantitative PCR using the iCycler iQ Real time PCR detection system and iQ SYBR Green supermix (Biorad, Hercules, CA) was performed, using primers for Cyclin D (forward 5'-GAAGTACCTGGACCGCTTCC-3'; reverse 5'-CCTTGCAGCTGCTTAGACG-3') and Axin 2 (forward 5'-TTATGCTTTGCACTACGTCCCTCCA-3'; reverse 5'-CGCAACATGGTCAACCCTCAGAC-3') and 18s rRNA (forward 5'-CGCCGCTAGAGGTGAAATTC-3', reverse 5'-TTGGCAAATGCTTTTCGCTC-3') as an endogenous control for normalization. The comparative C_T (DDCT) analysis method (Genex software, Biorad) was used to assess the relative fold changes in gene expression. The experiments were repeated in triplicate and the mean fold changes and standard error of the mean are reported.

3. Results and discussion

3.1. The role of the five conserved PPPS/TP sites in LRP6 signaling to β -catenin

Five highly conserved, proline-rich phosphoacceptor motifs (PPPS/TP) are present in the intracellular domain (ICD) of LRP5/6 (Fig. 1A). The positions of these five sites are: (1) S1490 (2) T1529, T1530 (3) T1572 (4) S1590 (5) S1607. Among them, the first motif appears to be critical for signal transmission, as suggested by the observation that a C-terminal truncated ICD of LRP6 can stimulate β -catenin when the first PPPS/TP is present in the context of a membrane-associated protein or when transferred to a heterologous receptor [11]. Furthermore, this first phosphoacceptor site, S1490, can be directly phosphorylated by GSK3- β *in vivo*, and is flanked by two conserved, Wnt-regulated CK1- γ activation motifs, whose phosphorylation is required for signal transduction [8]. In fact, stimulation of HEK-293T cells with Wnt3a induced the phosphorylation of S1490 within endogenous LRP6 (pLRP6^{S1490}) in control cells

transfected with GFP (Fig. 1B). The specificity of the immunodetection of pLRP6^{S1490} was confirmed by transfection of full length wild type LRP6 (LRP6-WT), which was constitutively phosphorylated in position S1490 when overexpressed, as recently reported [8], but not in cells transfected with a mutant LRP6 in which each serine/threonine residues in all PPPS/TP motif was replaced for alanine (LRP6-5A). We observed only a slight increase of pLRP6 in cells overexpressing LRP6-WT upon Wnt activation, and LRP6-5A did not interfere with the phosphorylation of the endogenous LRP6. Furthermore, LRP6-WT but not LRP6-5A stimulated the basal luciferase expression of a β -catenin reporter cell line, and led to a remarkable increase in the transcriptional response to Wnt3a in a dose-dependent fashion (Fig. 1C). The Wnt3a-stimulated β -catenin reporter expression was not affected by LRP6-5A (Fig. 1D). In line with these observations, control (not shown) and LRP6-5A expressing cells displayed partitioned β -catenin between low nuclear levels and cell-cell membrane accumulation. Wnt3a activation caused some increased nuclear β -catenin localization in LRP6-5A, while LRP6-WT transfected cells exhibited enhanced nuclear staining for β -catenin already under basal conditions, and almost exclusively nuclear localized β -catenin upon Wnt3a stimulation (Fig. 1E). In addition to transcriptional expression from the β -catenin reporter in response to Wnt, mRNA levels of Cyclin D1 and Axin2, both direct transcriptional targets of β -catenin [19], were upregulated in response to Wnt3A stimulation in cells expressing LRP6-WT, but LRP6-5A (Fig. 1F). This correlates with the activation of the β -catenin reporter in the presence of LRP6-WT but not LRP6-5A, suggesting this reporter is an appropriate assay for measuring Wnt3A stimulation of these cells.

3.2. The first PPPS/TP site has a limited role in LRP6-mediated β -catenin activation

As the contribution of the phosphorylation of S1490 in LRP6 to β -catenin signaling has been primarily studied in the context of chimeric truncated LRP6 ICD, we next mutated this site to alanine in the full length LRP6 and explored the functional consequences. Surprisingly, the full length LRP6 A1490 mutant (ATTSS) exhibited a limited ability to stimulate the β -catenin reporter under basal conditions (Fig. 2A). Moreover, the A1490 mutant transduced the Wnt-initiated signal efficiently, to an extent only slightly less than that of the LRP6-WT (Fig. 2B). Furthermore, an LRP6-5A mutant in which S1490 was re-introduced (SAAAA) was phosphorylated at that residue even under basal conditions, but was not able to transduce signals to β -catenin under basal or Wnt-stimulated conditions (Fig. 2A-B). We did not observe any enhanced immunodetection of pLRP6 above the endogenous levels in basal or Wnt-stimulated LRP6-A1490 mutant cells, which supported the high specificity of the immunodetection (Fig. 2C). These results indicated that LRP6 phosphorylation on residue S1490 is neither absolutely required nor sufficient for transducing the Wnt-initiated signal to β -catenin.

3.3. No individual PPPS/TP motifs are sufficient nor do they cooperate with the first PPPS/TP site for LRP6 signaling to β -catenin

These observations prompted us to investigate whether another PPPS/TP motif could play a more critical role in Wnt signaling through LRP6. To test this, we conducted a systematic add-back approach, in which each of the alanine residues was mutated back to its original serine or threonine residue. As shown in Fig. 3A-C, none of these LRP6 molecules harboring a single PPPS/TP motif were able to restore the function of LRP6-WT when overexpressed or stimulated with Wnt3A. We then asked whether LRP6 mutants having two PPPS/TP phosphoacceptor sites, including the first one, would function as LRP6-WT if effectively expressed and phosphorylated at serine 1490. However, none of the mutants exhibiting two potential phosphorylation motifs mediated signaling to β -catenin basally or when stimulated by Wnt3A (Fig. 3A-C). These data indicate that LRP6 requires more than two of these putative phosphorylation sites to retain its ability to transduce a downstream signal able to fully activate β -catenin.

3.4. The PPPS/TP motifs in LRP6 act in a combinatorial fashion

We next selectively mutated each of the native sites in a combinatorial fashion. As shown in Fig. 4A, only the LRP6 mutant lacking its second PPPS/TP motif (SATSS) behaved as the wild type LRP6, while mutation of the third and fourth PPPS/TP motif (STASS and STTAS) exhibited an impaired signaling capacity, nearly a third of that of the LRP6 WT. An LRP6 mutant lacking only its fifth PPPS/TP motif (STTSA) was also defective, stimulating the reporter system nearly 50% of that induced by Wnt through the LRP6-WT. This set of observations suggests that LRP6 requires PPPS/TP sites one, three, four and five for full β -catenin activation by Wnt.

Wnt activation results in the assembly of large protein complexes [10], whose size is close to that of the ribosomes and thus readily visualized as intracellular clusters, which appear to be initiated by the recruitment of Axin to phosphorylated LRP6 [20]. Thus, we took advantage of the availability of LRP6-WT and representative mutants lacking the second PPPS/TP motif (SATSS) that are effective in transducing Wnt signaling and the AAAAA mutant that is signaling defective, to explore whether these particular phosphoacceptor motifs regulate formation of Axin containing complexes. Whereas the ability of LRP6 to co-immunoprecipitate endogenous or a transfected AU5-tagged form of Axin upon Wnt stimulation was quite limited and variable (not shown), we noticed that upon activation of cells expressing LRP6-WT, the intracellular localization of Axin changes rapidly from diffuse and cytosolic to aggregated Axin-containing clusters, like those recently described (Fig. 4B) [20]. Similar results were obtained in cells expressing a signaling competent LRP6 mutant (SATSS), but no relocation of Axin was observed in cells expressing LRP6 signaling defective mutant (AAAAA). The percentage of WT expressing cells containing Axin clusters was comparable to the percentage in cells expressing the signaling competent mutant (SATSS) when stimulated with Wnt3A, but no change was observed after Wnt3A stimulation in cells expressing the signaling defective LRP6 mutants (AAAAA, Fig 4C, and its single phospho-acceptor site mutants, not shown). These findings suggest that the presence of at least 4 PPPS/TP motifs is necessary to retain the full signaling capacity of LRP6, which is reflected in its ability to promote Axin redistribution and nuclear β -catenin signaling (Fig 4D).

Overall, our present findings indicate that whereas a full length LRP6 protein with only one, or even two functional PPPS/TP motifs is not sufficient to induce canonical Wnt signaling, four of the PPPS/TP motifs in the ICD of LRP6 (sites 1, 3, 4 and 5) must be present for full induction of β -catenin, and that site 2 is dispensable. We expect that our present results will provide a strong rationale for the future development of immunological tools enabling the precise kinetic analysis of the potential phosphorylation of each of the four critical PPPS/TP motifs. In this regard, whereas GSK3 β specifically phosphorylates LRP6 at its first PPPS/TP motif, it is possible that this or additional Wnt-regulated kinases may contribute to the regulation of the additional critical phosphoacceptor sites [8].

In conclusion, our data suggest that no individual PPPS/TP motif in LRP6 is alone sufficient to promote β -catenin activation. Furthermore, the use of full-length LRP6 mutants harboring multiple mutations in its five PPPS/TP sites revealed that the five PPPS/TP sites are not functionally equivalent, and that they each may contribute in a combinatorial fashion to LRP6 function in the canonical Wnt- β -catenin pathway. These observations raise the possibility that multiple kinases may need to be engaged upon Wnt binding to LRP6 and Frizzled to activate β -catenin signaling, which may explain the difficulty in dissecting the precise mechanism by which Wnt regulates LRP6 to ultimately signal to the nucleus through β -catenin.

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Fig 1. Mutation of the five putative phosphorylation sites on the ICD of LRP6 abolishes β -catenin activation by Wnt

(a) LRP6 contains five PPPS/TP domains in its intra-cellular domain. (b) HEK-293T cells transfected with GFP, LRP6-WT or LRP6-5A were functionally analyzed for LRP6 phosphorylation. Cells lysates were probed for pLRP6^{S1490}, V5 and tubulin. SE: Short exposure. The longer exposure (LE) reveals endogenous LRP6 proteins. (c) LRP6 constructs were functionally compared using a luciferase based reporter assay after the cells were stimulated with serial dilutions of Wnt3a conditioned media for 8 hours. Data shown are representative of at least three independent experiments. (d) Negative controls GFP and LRP6-5A are shown at minimum and maximum Wnt3a conditioned media dilutions shown in panel C. (e) LRP6-WT and LRP6-5A are both tested with immunofluorescence for their abilities to induce nuclear localization of β -catenin (green). DAPI-nuclei staining of the same field is shown in the inserts (blue). (f) mRNA levels of two transcriptional targets of β -catenin, Cyclin D1 and Axin 2, were measured by quantitative RT-PCR in cells expressing GFP, LRP6-WT or LRP6-5A and stimulated with either control or Wnt3A conditioned media.

Fig 2. Phosphorylation of LRP6 on S1490 is not sufficient for full basal and Wnt-stimulated β -catenin activation

(a) Full length LRP6 mutants either lacking a functional site 1, or containing only a functional site 1 (no other functional PPPS/TP motifs) were analyzed for luciferase activity. The same constructs were evaluated following overexpression alone or (b) Wnt3a stimulation. (c) Similar samples were subjected to pLRP6^{S1490}, V5 and tubulin western-blots. All data are representative of at least three independent experiments.

Fig 3. No single PPPS/TP motif is sufficient for LRP6 signaling to β -catenin, and each plays only a limited cooperative role with the first PPPSP phosphoacceptor site

The single add-back series of LRP6 mutants were designed so that only one of the five PPPS/TP motifs would be present in the full-length protein. The double add-back series of LRP6 mutants were designed so that S1490 was always present (site 1) with one additional site. These mutants were functionally analyzed in a luciferase assay when treated with (a) control or (b) Wnt3a conditioned media. (c) Western blots to demonstrate phosphorylation status of S1490 of various LRP6 mutants when stimulated with control or Wnt3a conditioned media. All data are representative of at least three independent experiments.

Fig 4. PPPS/TP sites 1, 3, 4, and 5 contribute to LRP6/Axin complex formation and β -catenin activation

(a) A final series of five mutants was designed in which one site was selectively mutated and four functional sites remained. These constructs were tested for luciferase activity in response to Wnt3a conditioned media, and their expression in 293T cells is shown. (b) Selected LRP6 constructs were tested for their abilities to induce changes in Axin (green) localization when overexpressed or stimulated with Wnt3a conditioned media. All data are representative of at least three independent experiments. (c) Staining of cell in panel C was quantified by counting the percentage of cells stained for expression of LRP6-WT, AAAAA, or SATSS that contained Axin clusters. (d) In a model for LRP6 function, four of the sites are required for β -catenin activation, and site 2 is dispensable.