Wnt Signal Amplification via Activity, Cooperativity, and Regulation of Multiple Intracellular PPPSP Motifs in the Wnt Co-receptor LRP6

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Low density lipoprotein receptor-related protein 6 (LRP6) and its homologue LRP5 serve as Wnt co-receptors that are essential for the Wnt/β-catenin pathway. Wnt activation of LRP6 leads to recruitment of the scaffolding protein Axin and inhibition of Axin-mediated phosphorylation/destruction of β-catenin. We showed that five conserved PPPSP motifs in the LRP6 intracellular domain are required for LRP6 function, and mutation of these motifs together abolishes LRP6 signaling activity. We further showed that Wnt induces the phosphorylation of a prototypic PPPSP motif, which provides a docking site for Axin and is sufficient to transfer signaling activity to a heterologous receptor. However, the activity, regulation, and functionality of multiple PPPSP motifs in LRP6 have not been characterized. Here we provide a comprehensive analysis of all five PPPSP motifs in LRP6. We define the core amino acid residues of a prototypic PPPSP motif via alanine scanning mutagenesis and demonstrate that each of the five PPPSP motifs exhibits signaling and Axin binding activity in isolation. We generated two novel phosphorylation-specific antibodies to additional PPPSP motifs and show that Wnt induces phosphorylation of these motifs in the endogenous LRP6 through glycogen synthase kinase 3. Finally, we uncover the critical cooperativity of PPPSP motifs in the full-length LRP6 by demonstrating that LRP6 mutants lacking a single PPPSP motif display compromised function, whereas LRP6 mutants lacking two of the five PPPSP motifs are mostly inactive. This cooperativity appears to reflect the ability of PPPSP motifs to promote the phosphorylation of one another and to interact with Axin synergistically. These results establish the critical role and a common phosphorylation/activation mechanism for the PPPSP motifs in LRP6 and suggest that the conserved multiplicity and cooperativity of the PPPSP motifs represents a built-in amplifier for Wnt signaling by the LRP6 family of receptors.

The canonical Wnt/β-catenin pathway controls cell proliferation and cell fate during embryogenesis and adult tissue homeostasis, and mutations that disrupt Wnt signaling contribute to a variety of diseases including cancer and osteoporosis (1). In the absence of an extracellular Wnt ligand, cytoplasmic β-catenin is phosphorylated and degraded by a complex that includes the scaffolding protein Axin, tumor suppressor protein APC, and the kinases GSK3β and CKI, preventing β-catenin-activated transcription in the nucleus (2). The canonical pathway is initiated when a Wnt ligand binds to a member of the Frizzled serpentine receptor family and its co-receptor low density lipoprotein receptor-related protein-related protein 6 (LRP6) or a close relative such as LRP5 (3, 4). This Wnt-induced Fz-LRP6 complex recruits Axin to the plasma membrane (5–7) and results in the inhibition of β-catenin phosphorylation and degradation. This leads to β-catenin accumulates in the cytoplasm and translocation into the nucleus to bind the LEF/TCF transcription factor and activates the transcription of Wnt target genes (1, 8). LRP5 and LRP6 are of critical importance in human diseases. Loss-of-function and gain-of-function mutations in LRP5 result in osteopetrosis-pseudoglioma and high bone mass disease, respectively (9–11). Recently a LRP6 mutation has been shown to be associated with coronary artery disease and osteoporosis (12). These LRP5 and LRP6 mutations result in abnormal Wnt/β-catenin signaling, which likely underlies the pathogenesis of these disorders.

LRP5/6 functions in the activation of the Wnt pathway via the recruitment of Axin to the plasma membrane (5, 6). The LRP6 cytoplasmic domain is essential for Axin binding, and its deletion in a LRP6 mutant, LRP6ΔC, results in a dominant negative receptor that binds Wnt but is unable to bind Axin (6, 13). The LRP6 extracellular domain has auto-inhibitory activity because its deletion in LRP6ΔN results in a constitutively activated receptor that binds Axin in the absence of Wnt ligand (5, 6). In a previous study we identified a conserved PPPSP motif, which is reiterated five times in LRP5, LRP6, and their Drosophila homologue Arrow, as the docking site for Axin-binding (6). We showed that a prototypic PPPSP motif (motif A) (supplemental Fig. S1) functions as a module that is sufficient to transfer signaling activity to a heterologous receptor, in this case a truncated LDL receptor (LDLRΔN) (6). We further showed

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that this prototypic PPPSP motif is phosphorylated and is capable of binding Axin in a phosphorylation-dependent manner (6). In a following study we determined that this prototypic PPPSP site is phosphorylated by GSK3, which primes the phosphorylation of a neighboring S residue at the +3 position (PPPSPXS) by CKI, resulting in maximal Axin binding (14). In the full-length LRP6 receptor we found that mutations of all five PPPSP motifs (LRP6m5) results in a dominant negative receptor that is unable to bind Axin and thus is analogous to LRP6ΔC (6, 14). Although these studies imply the importance of PPPSP motifs in LRP6 function, the activity and regulation of each of these PPPSP motifs and the relationship among them in mediating Wnt/LRP6 signaling have not been characterized and are the subjects of this study.

EXPERIMENTAL PROCEDURES

Plasmids—All LRP6, LRP6ΔN, and LDLRΔN constructs were tagged with the vesicular stomatitis virus G (VSVG) epitope tag and cloned into pCS2+ as previously described (6). For PPPSP motif transfer into LDLRΔN, the last 11 amino acids of LDLRΔN (residues 781–860) were replaced with PPPSP motifs of 15–25 amino acid residues from LRP6. Transferred residues from LRP6 are: A (resides 1483–1497), B (resides 1521–1541), C (resides 1562–1586), D (resides 1580–1600), and E (resides 1596–1613). Single amino acid substitutions were generated using a QuikChange mutagenesis kit (Stratagene). LRP6 full-length and LRP6ΔN were tagged with the vesicular stomatitis virus G (VSVG) epitope tag and cloned into pCS2 (6). Although these studies imply the importance of PPPSP motifs in LRP6 function, the activity and regulation of each of these PPPSP motifs and the relationship among them in mediating Wnt/LRP6 signaling have not been characterized and are the subjects of this study.

For PPPSP motif transfer into LDLR

transfected with an Axin-Flag plasmid and cultured until stage 25 for marker gene analysis. Semi-quantitative reverse transcription-PCR was performed as previously described (16). Anterior and posterior marker genes Otx2, En2, Krox20, HoxB9 (also known as XLHbox6), and EFlα were amplified using primer sequence obtained through the De Robertis lab home page or were previously used in another study (16).

RESULTS

Ala-1490 mutant (Ab1490) was used as previously described (6). The LRP6 antibody to the phosphorylated A motif (Ab1490) was used as previously described (6). The LRP6 C motif phospho-specific antibody (Ab1572) was produced by Covance (Denver, PA) using a phosphopeptide CEVPVPPPT*PRSQY (indicates phosphorylated residue) conjugated to keyhole limpet hemocyanin. Ab1607 was raised to the phosphopeptide LRP6 pE KHLYPPPS*PCTDSS. The bleeds were passed through a phosphopeptide column, followed by at least two passes through a non-phosphopeptide column and concentrated to 150 mg/ml. For co-IP experiments, GSK3β was co-transfected to increase PPPSP site phosphorylation, and anti-FLAG (M2) was used to precipitate Axin. Cre-mediated deletion of Gsk3α was performed in mouse embryonic fibroblasts (MEFs) derived from Gsk3β+/−, Gsk3αflx/flx mice as previously described (14, 15).

Xenopus Animal Cap Assay—Xenopus laevis embryos were injected with mRNAs at the two-cell stage in the animal pole. Animal explants (animal caps) were dissected from stage 9 embryos and cultured until stage 25 for marker gene analysis. Semi-quantitative reverse transcription-PCR was performed as previously described (16). Anterior and posterior marker genes Otx2, En2, Krox20, HoxB9 (also known as XLHbox6), and EFlα were amplified using primer sequence obtained through the De Robertis lab home page or were previously used in another study (16).

In our previous studies we showed that a 15-amino acid region of LRP6 encompassing the first PPPSP motif, referred to as motif A, is fully active when transferred to the truncated LDLR (LDLRΔN) in the absence of any Wnt ligand (6). In this context we demonstrated that the PPPSP motif is phosphorylated, producing a distinct more slowly migrating band in electrophoresis followed by immunoblotting, and this phosphorylated PPPSP motif is specifically co-immunoprecipitated (co-IPed) with Axin (6). To investigate which residues might be critical for phosphorylation and activation of the Wnt/β-catenin pathway, we performed an alanine mutation scan (replacing each amino acid residue with alanine) using the prototypic LDLRΔN-PPPSP construct (6), which is referred to here as LDLRΔN-A to distinguish it from other PPPSP motifs (B to E); for residue Ala1490, glycine replacement was performed instead (Fig. 1A). We first examined the protein expression using an antibody to the VSVG tag and determined that they were expressed at comparable levels (Fig. 1B). In immunoblotting most of the LDLRΔN-A mutants exhibited a slower mobilizing band, which migrated similarly as the phosphorylated wild type (WT) LDLRΔN-A and was detected by the phospho-specific antibody Ab1490 (Fig. 1B, asterisks), although this upper band was greatly reduced in P1488A mutant. All LDLRΔN-A mutants, like the WT LDLRΔN-A, exhibited a more quickly migrating and presumably unphosphorylated band that differed in its position depending on the position of the alanine replacement. Consistent with our previous reports (6, 14), S1490A and P1491A mutants did not display the slow migrating upper band and were not reactive to Ab1490. Therefore only the SP residues appear to be essential for phosphoryl-
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FIGURE 1. Alanine scanning mutagenesis of the LRP6 motif A. A, the 15-amino acid region straddling motif A that was transferred to the LDLRN receptor. The underlined residues were subjected to alanine substitution at each position or, in the case of Ala1492, to glycine substitution. Motif A site was also altered to resemble motif B or C by substituting Ser1490 with threonine and motif D by substituting Pro1487 with cysteine. B, immunoblotting of lysates from 293T cells expressing VSVG tagged LDLR mutants. The upper band was recognized by the LRP6 pA (Ab1490) phospho-specific antibody (asterisks). C, cytoplasmic β-catenin levels of the LDLRN-A mutants. The immunoblot of purified cytoplasmic proteins was probed for β-catenin and α-tubulin (loading control). The circled P denotes the Ser1490 position, and brackets delineate the core seven amino acids. D, activity of the LDLRN-A mutants using the TOPFLASH reporter assay. Relative luciferase activity of the five intracellular PPPSP motifs of LRP6 reveals the consensus of PPP(S/T)P-S motif had only minimal or small effects on the activity of the motif, with the exception of N1486A, which produced a hyperactive effect (Fig. 1D). Using the WT LDLRN-A for comparison, we observed a significant reduction in activity for the alanine (or glycine) replacement of each of the core seven amino acid residues corresponding to PPPSPAT (Fig. 1D). Alanine replacements outside the core PPPSPXS motif had only minimal or small effects on the activity of the motif, with the exception of N1486A, which produced a hyperactive effect (Fig. 1D). Examination of the “B- or C-like” variant S1490T demonstrates that a Thr substitution of Ser activated the TOPFLASH as effectively (Fig. 1D), consistent with Ser/Thr conservation/equivalence at this position (Fig. 2A). However, the “D-like” P1487C substitution greatly diminished the activity similar to P1487A (Fig. 1D), indicating that a cysteine at this position (CPPSP) reduces the activity of the prototypic motif A. To validate our TOPFLASH reporter results, we measured the cytoplasmic β-catenin levels in cells expressing these LDLRN-A variant constructs (Fig. 1C). The increase in cytoplasmic β-catenin protein level corresponded well with the level of TOPFLASH activity, substantiating the importance of the core PPPSPXS motif.

Functional Comparison of Individual PPPSP Motifs—Alignment of the five PPPSP motifs of LRP6 reveals the consensus of PPP(S/T)PX(S/T) (Fig. 2A), consistent with our experimental evidence from mutagenesis of motif A. However, the surrounding amino acids at each motif are unique and in some cases

FIGURE 2. Comparison of each PPPSP motif transferred to LDLRN-A. A, alignment of the five LRP6 PPPSP motifs. The LDLRN-B, C, D, and E constructs were generated by the same methods used to make LDLRN-A. B, immunoblotting of the LDLRN-B, C, D, and E proteins expressed in 293T cells. Alanine substitutions at the Ser/Thr position (analogous to S1490A) of each PPPSP motif were also expressed in 293T cells for comparison. Note that no upper bands were resolved in LDLRN-C, LDLRN-D, and LDLRN-E. C, phosphorylation of the LDLRN-B and mutants. Immunoblotting of lysates from cells expressing LDLRN-B constructs. The upper band was abolished in the T1530A and P1531A mutants, similar to S1490A and P1491A in LDLRN-A. D, TOPFLASH reporter assay. LDLRN-C, LDLRN-D, and LDLRN-E were inactivated with the alanine substitution of the Ser/Thr residue similar to LDLRN-A S1490A mutation. E, LDLRN-B mutants T1530A, P1531A, and T1529A,T1530A (TT→AA) lowerd but did not abolish activity.
evolutionarily conserved (supplemental Fig. S1). To directly examine whether the other four PPPSP motifs have activities similar to the prototypic motif A, we transferred a short stretch of amino acid sequence spanning the LRP6 B, C, D, and E motifs to LDLRΔN, using the same approach as the LDLRΔN-A construct along with the alanine replacement of the Ser/Thr residue in each of the PPP(S/T)P motif. We examined the expression of LDLRΔN-B, -C, -D, and -E by immunoblotting and found that only the LDLRΔN-B construct yielded a more slowly migrating band, which presumably reflects phosphorylation (Fig. 2B). We suspected that LDLRΔN-C, -D, and -E were phosphorylated, but the phosphorylation in these contexts did not alter mobility during electrophoresis (see below). Motif B differs from the others by containing two internal threonines, although only the second (T1530) is conserved in the fly homologue Arrow (supplemental Fig. S1, arrowhead). To determine which threonine correlates with the upper band in LDLRΔN-B and whether motif B works similarly as motif A, we made alanine mutations at each of the underlined residues PPTTPCS (Fig. 2C).

We found that T1530A and P1531A mutants abolished the upper band, analogous to the SP mutations in LDLRΔN-A, generally low (14), LDLRΔNm5 expresses well, as do LRP6ΔN-A, -B, -C, -D, and -E (Fig. 3C). We tested the activity of these LRP6ΔN constructs and found that LRP6ΔN-A, -B, -C, and -E strongly activated the TOPFLASH reporter, whereas the LRP6ΔN-D was significantly weaker (Fig. 3A). These results are fully consistent with those derived from the motif transfer to LDLRΔN and indicate that in isolation motifs A, B, C, and E are highly active, whereas motif D is significantly less active, in two distinct contexts in the stimulation of the Wnt/β-catenin pathway.

We have previously demonstrated that LRP6ΔN is highly phosphorylated, resulting in multiple slower mobilizing bands that can be reduced with phosphatase treatment (6). We examined the LRP6ΔN-A single active motif using VSVG antibody and an observed 33-kDa band that corresponds to the predicted molecular mass and two prominent upper bands at 40 and 47 kDa (Fig. 3B). Phosphatase treatment of LRP6ΔN-A reduced the 47 kDa down to a faster migrating species and removed phosphorylation at the A motif as assayed by Ab1490.

To determine whether the observed TOPFLASH activity correlated with Axin binding, we examined the binding

Using the TOPFLASH reporter assay we found that LDLRA-A, -B, -C, and -E had comparable or greater signaling activities when compared with LDLRA-A (Fig. 2B). LDLRA-D had the weakest activity (Fig. 2B), consistent with the result from the D-like LDLRA-A P1487C mutant (Fig. 1, C and D). Mutation of the Ser/Thr residue inactivated each of the transferred PPPSP motifs (Fig. 2D), with the exception of motif B, whose mutation diminished but did not abolish its activity even when both Thr1529 and Thr1530 were simultaneously mutated to alanine (Fig. 2E).

We showed previously that deletion of the LRP6 extracellular region (LRP6ΔN) results in a constitutively active receptor and that mutation of the Ser/Thr residue in all five PPPSP motifs (LRP6ΔNm5) abolishes its activity and prevents Axin binding (6, 14). To independently compare the five PPPSP motifs in the context of the LRP6 intracellular region, we selectively restored the Ser/Thr residue in each of the five PPPSP motifs in LRP6ΔNm5. For example, LRP6ΔN-A contains only the active motif A, whereas the other four motifs remain mutated. We have noted previously that although LRP6ΔN expression levels are whereas the T1529A mutant had no effect on the upper band (Fig. 2C).
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between Axin (FLAG-tagged) and each of LRP6ΔN-A, -B, -C, -D, and -E via co-IP. In these experiments LRP6ΔN-A, LRP6ΔN-B, and LRP6ΔNm5 were expressed at similar levels, whereas LRP6ΔN-C, LRP6ΔN-D, and LRP6ΔN-E were present at higher levels, and LRP6ΔN was expressed at a significantly lower level (Fig. 3C). Axin co-IPed LRP6ΔN strongly (lane 5) and each of LRP6ΔN-A, -B, -C, -D, and -E (lanes 6–10), but not LRP6ΔNm5 (Fig. 3C, lane 11). Axin co-IPed significantly less LRP6ΔN-D than LRP6ΔN-C and -E (lane 9 versus lanes 8 and 10), demonstrating that the weak signaling activity of LRP6ΔN-D correlates with poor Axin binding. We note that LRP6 intracellular domain is highly enriched with Ser/Thr residues (28%) and is heavily phosphorylated both within and outside PPPSP motifs (i.e. even when all Ser/Thr residues in PPPSP motifs are mutated (14)). The smearable and multiple bands of LRP6ΔN and mutants are primarily caused by the heavy phosphorylation (6, 14) (Fig. 3B).

We further tested LRP6ΔN-A and -D activities in anterior-posterior patterning in X. laevis embryos. It is well established that graded Wnt/β-catenin signaling causes gradual posteriorization (18, 19), which can be assayed via the expression of endogenous anterior-posterior markers. We used noggin (a BMP inhibitor) to induce anterior neural tissue in animal pole explants, as shown by the expression of an anterior marker Otx2 (supplemental Fig. S2, compare lanes 1 and 10). Injection of Wnt3a mRNA caused posteriorization, as demonstrated by the suppression of Otx2 and induction of Krox20 and HoxB9, a hindbrain and spinal cord marker, respectively (supplemental Fig. S2, compare lanes 1 and 9). LRP6ΔN mRNA had similar activity and shifted the cell fate to posterior in a dose-dependent manner (supplemental Fig. S2, lanes 2–5). We observed that LRP6ΔN-A shifted the cell fate to express posterior genes En2, Kro20 and HoxB9 while suppressing Otx2 expression (supplemental Fig. S2, lane 6). LRP6ΔN-D induced the expression of the posterior genes; however, it failed to suppress the anterior marker Otx2 (supplemental Fig. S2, lane 7), indicating that LRP6ΔN-D is also less active than LRP6ΔN-A in the Xenopus embryo.

Generation of Phospho-motif C and -motif E Antibodies—Our study thus far suggests that each of the five PPPSP motifs exhibits signaling activities similar to the prototypic motif A that we initially characterized (6), although signaling strength varies. Given the functional importance of the Ser/Thr residue in these PPPSP motifs, it seems likely that Ser/Thr phosphorylation, like what we have observed in motif A (6, 14), is also critical for the activity of these other PPPSP motifs. Although we were only able to observe the phosphorylation-associated mobility shift for motifs A and B, but not for C, D, or E (Fig. 2B), it remains possible that phosphorylation of motifs C, D, or E nonetheless occurs but does not cause mobility changes that were observable in the context of LDLRΔN. We previously generated Ab1490, which specifically recognizes PPPSP phosphorylation in motif A (6). We therefore attempted to generate antibodies that specifically recognize PPPSP phosphorylation in other motifs, using synthetic phosphopeptides as immuno- gens (see “Experimental Procedures”). We were able to derive Ab1572 and Ab1607, which were designed to recognize the phosphorylated motif C and motif E, respectively. In peptide dot blotting, Ab1572 recognized phospho-motif C peptide (pC) but not the unphosphorylated control peptide (supplemental Fig. S3A). Likewise we found that Ab1607 only recognized the phospho-motif E peptide (pE) but not the control peptide (supplemental Fig. S3B). We found that Ab1572 also bound weakly to pE, although this binding was rather minor in comparison with pC binding (supplemental Fig. S3A). We attempted to remove pE cross-reactivity from Ab1572 via absorption through a pE peptide column, but unfortunately such pE-absorbed Ab1572 no longer recognized the pC peptide (data not shown).

We tested Ab1572 and Ab1607 using the constitutively active LRP6ΔN and observed a robust signal that was absent in LRP6ΔNm5 (Fig. 4A, compare lanes 2 and 8), suggesting strong phosphorylation of motifs C and E in LRP6ΔN. Interestingly, Ab1572 reacted with LRP6ΔN-C and -E, but not LRP6ΔN-A, -B, or -D, whereas Ab1607 reacted only with LRP6ΔN-E, but not LRP6ΔN-A, -B, -C, or -D (Fig. 4A). These results not only demonstrate that LRP6ΔN-C and -E are phosphorylated at the respective PPPSP motif but also suggest that Ab1607 is highly specific for motif E phosphorylation, whereas Ab1572 is spe-
specific for phosphorylation at motifs C and E, similar to the specificity observed in peptide dot blotting (supplemental Fig. S3A).

**Wnt Induces Motifs C and E Phosphorylation via GSK3**—Our previous studies have shown that motif A in the wild type LRP6 is rapidly phosphorylated in response to Wnt stimulation (6, 14). Using the new phospho-specific Ab1572 and Ab1607, we observed that motif E, and most likely motif C as well, became rapidly phosphorylated upon stimulation by Wnt3α-conditioned medium in MEFs (Fig. 4B). Similar to motif A phosphorylation detected by Ab1490 (6, 14), phosphorylation at motifs C and E were detectable within 5 min of Wnt3α treatment, increased with Wnt exposure time and lasted for at least 4 h (Fig. 4B). We have shown previously that motif A phosphorylation is mediated by GSK3 (14). We therefore tested whether motifs C and E were also phosphorylated by GSK3 by using MEFs derived from Gsk3β−/−, Gsk3αlox/lox mice. In these experiments, all of the transfected full-length LRP6 constructs were expressed at comparable levels in 293T cells. For better comparison, the activity of the WT LRP6 alone was set to 1. C and D, TOPFLASH reporter activity of LRP6 mutants with two mutated PPPSP motifs (i.e. with three active motifs). C, LRP6 mutants alone. D, LRP6 mutants with co-transfected Wnt1. All of the LRP6 double mutants were inactive except for LRP6 ab, which retained some activity. LRP6 cd and LRP6 ce displayed dominant negative activity in the presence of Wnt1. E, Axin binding abilities of the full-length LRP6 and single motif mutants. LRP6 single mutants at motif C, D, or E had significantly reduced Axin binding abilities.
MEFs infected with a control lentivirus containing GFP, phosphorylation of the endogenous LRP6 at motifs C and E was induced in response to Wnt3a conditioned medium (Fig. 4C). However, in MEFs infected with a viral vector expressing the Cre recombinase, which deleted the Gsk3α gene from the genome and significantly reduced the Gsk3α protein level, we observed marked reduction in Wnt3a-induced phosphorylation at motifs C and E (Fig. 4C). The residual phosphorylation present after Cre expression is likely due to incomplete deletion of the two Gsk3α alleles and/or the long half-life of Gsk3α mRNA or protein. Collectively our data suggest that Wnt induces PPPSP phosphorylation similarly at motifs A, C, and E, and the phosphorylation of these related motifs are likely mediated via GSK3.

Cooperativity of PPPSP Motifs in LRP6—Our characterization of the PPPSP motifs reveals that each individual motif, when added back to LRP6ΔN m5 or transferred to LDLRΔN, can bind to Axin and activate the Wnt/β-catenin pathway, suggesting that a single PPPSP motif in these contexts is sufficient for Wnt signaling activation. However, the fact that five PPPSP motifs are conserved in sequence and spacing arrangement in LRP6, LRP5, and their homologues from fruit flies to human (supplemental Fig. S1) suggests that in the context of the full-length LRP6 receptor PPPSP motif, multiplicity is important for Wnt signaling, possibly via functional cooperation. Indeed two copies of motif A in tandem provides more robust signaling than a single copy when transferring to LDLRΔN (6). To determine whether a single PPPSP motif in the full-length LRP6 is sufficient to activate Wnt signaling, we selectively restored one PPPSP motif in LRP6 m5, which harbors the Ser/Thr to alanine substitution in all five PPPSP motifs in the full-length LRP6 and is functionally inactive (6). For example, LRP6-A contains only the active motif A, and the other four motifs are mutated. Using the TOPFLASH reporter assay, we found, surprisingly, that each of the LRP6 single motif receptors behaved indistinguishably from LRP6 m5 in that each not only failed to activate Wnt signaling but also behaved as dominant negative receptors, i.e. blocked signaling by co-expressed Wnt1 (Fig. 5, A and B). These results indicate that a single PPPSP motif is not sufficient to signal in the context of the full-length LRP6.

To further understand the interdependence of each PPPSP motif in LRP6, we selectively mutated a single motif in the full-length LRP6, leaving four active PPPSP motifs. For example, LRP6 a indicates that only motif A is mutated (by Ser to Ala substitution in PPPSP). These “single mutant” receptors tested the necessity of each motif in the full-length LRP6. In the absence of co-transfected Wnt, we found that the activity of LRP6 b was slightly reduced, whereas that of LRP6 a, c, d, and e single mutant receptors was significantly reduced compared with that of the WT LRP6 (Fig. 5A). The weak effect of LRP6 b prompted us to delete the entire conserved region of motif B (APPTPCSTDVCD), and we found that this LRP6 deletion mutant behaved comparably with LRP6 b (data not shown). Next we examined these single-motif LRP6 mutants in the presence of Wnt1. The WT LRP6 and Wnt1 synergized strongly to activate the TOPFLASH reporter (Fig. 5B). In the presence of Wnt1, LRP6 b retained the highest activity followed closely by LRP6 a, whereas the ability of LRP6 c, LRP6 d, and LRP6 e to synergize with Wnt1 was significantly reduced, with each displaying less than half of the WT LRP6 activity (Fig. 5B).

Because motif D in isolation displayed the lowest activity and weakest Axin binding, it was unexpected that its mutation in the full-length receptor would dramatically reduce the activity of LRP6. One likely explanation is that the PPPSP motifs cooperate in the full-length receptor. To further investigate the mutual dependence of these motifs, we generated a series of “double mutant” LRP6 to examine the activity from the remaining three intact PPPSP motifs. For example, LRP6 ab harbors the Ser/Thr to alanine substitution in motifs A and B. Almost all of the LRP6 double mutants were inactive and failed to synergize with Wnt1 with the exception of LRP6 ab, which contains active C, D, and E motifs (Fig. 5, C and D). LRP6 ab synergized with Wnt1, exhibiting activity lower than the WT LRP6 but higher than LRP6 c, LRP6 d, and LRP6 e single mutant receptors (Fig. 5D). Nonetheless, either motif A or motif B still displayed synergy with the other motifs because mutation of either A or B in combination of mutation of C or D or E rendered LRP6 inactive (Fig. 5D). Two of the LRP6 double mutants, LRP6 cd and LRP6 ce, exhibited dominant negative activity by repressing the TOPFLASH reporter levels below that of Wnt1 alone, again demonstrating the importance of motif C and suggesting a strong cooperativity with the D and E motifs. The results together demonstrate that (i) the multiplicity (minimally four copies) of PPPSP motifs are essential for LRP6 function and (ii) motifs C, D, and E, which are clustered within the last 40 amino acids in the carboxyl terminus, are particularly critical for LRP6 function.

To correlate LRP6 signaling activity with Axin binding, we performed Axin co-immunoprecipitation experiments with LRP6 single mutant receptors using the WT LRP6 and LRP6 m5 as positive and negative controls, respectively. Although LRP6 m5 did not bind to Axin as previously demonstrated (14), LRP6 a and LRP6 b showed Axin binding comparable to that of WT LRP6, whereas LRP6 c, LRP6 d, and LRP6 e displayed significantly reduced binding to Axin (Fig. 5E). Therefore LRP6 signaling strength largely correlates with the Axin binding activity.

DISCUSSION

A key step in the activation of the Wnt/β-catenin pathway is Wnt-induced phosphorylation of LRP6 and LRP6-Axin interaction (5, 6). Previously we identified a PPPSP motif, which is reiterated five times (motifs A to E) in LRP6, LDLR, and their Drosophila homologue Arrow, as a functional module for Axin binding and signaling (6). However, our studies are largely based on the analysis of a single prototypic motif A, and the activity and regulation of other PPPSP motifs and their functional relationships have only been inferred or speculated but not yet investigated. In the present study we have extensively analyzed all five PPPSP motifs and report the following findings: (i) Using alanine scanning mutagenesis we confirmed that the core of this motif is composed of PPPSPXS, whereas amino acid residues outside this core appear to be less critical; (ii) we showed that each of the five PPPSP motifs in isolation is able to function as a signaling module in two distinct contexts, i.e. to transfer Wnt signaling activity to a heterologous LDLRΔN receptor and to restore signaling activity to the inactive
LRP6ΔN5 mutant receptor; (iii) we generated new phospho-specific antibodies to motifs C and E and showed that similar to motif A, motifs C and E of the endogenous LRP6 are phosphorylated in response to Wnt stimulation in a GSK3-dependent manner; (iv) we illustrated the necessity of each PPPSP motif and their synergistic activities in the full-length LRP6. Specifically, we demonstrated that LRP6 mutants lacking a single PPPSP motif have weakened signaling ability, whereas LRP6 mutants lacking two of the five PPPSP motifs are mostly inactive; and (v) we established that Axin binding directly correlates with the signaling strength in the context of either a single PPPSP motif in isolation or the full-length LRP6. These results together uncover similar activities and common regulation of all five PPPSP motifs and reveal the critical requirement for their synergistic cooperation in LRP6 signaling function.

Despite the commonalities, these PPPSP motifs nonetheless exhibit some noticeable differences both in isolation and in the full-length LRP6. Among isolated PPPSP motifs, we consistently observed that motif D displays the weakest activity. This is reflected by low TOPFLASH activity of LDLRAN-N (motif D transfer), LDLRAN-D (single motif D), and the D-like LDLRAN-A(P1487C) substitution. LDLRAN-D also exhibits less Axin binding and fails to suppress anterior marker Otx2 expression in Xenopus embryo explants. A key difference between motif D and the other motifs is its CPPSP composition, although the cysteine is conserved in LRP5 and Arrow (supplemental Fig. S1). Interestingly, however, motif D in the full-length LRP6 appears to be among the most critical ones, because its single mutation or any double mutation combining D and another motif results in a LRP6 with severely compromised or fully diminished activity. On the contrary, motif B exhibits strong signaling activity in isolation but had relatively weak contribution to the full-length LRP6, because its single mutation only results in slightly reduced LRP6 activity. However, motif B clearly cooperates with other motifs because double mutations combining B and another motif results in severely defective LRP6 signaling (Fig. 5, C and D). Our results in sum suggest that the order of activities of PPPSP motifs is E > A = B = C >> D in isolation, but C > D = E > A > B in the full-length LRP6.

Our phosphorylation-specific antibodies, Ab1490, Ab1572, and Ab1607, have provided direct evidence of Wnt-induced phosphorylation of LRP6 at motifs A (6, 14), C, and E (this study). Like the case for motif A, GSK3 appears to mediate Wnt-induced PPPSP phosphorylation at motifs C and E (Fig. 4C). We have not been able to raise antibodies that are specific for PPPSP phosphorylation at motifs B and D. However, motif B is phosphorylated, at least in isolation, because LDLRAN-B, like the characterized LDLRAN-A (6), displays in electrophoresis a more slowly migrating band that is abolished when PPTTP (motif B) is changed to PPTAP or PPTTA (Fig. 2C). Thus we have demonstrated direct evidence that three, and possibly four, of the five PPPSP motifs are phosphorylated. Although LDLRAN-D does not display a more slowly migrating band that we can correlate with phosphorylation (Fig. 2B), such a band is observed in the D-like LDLRAN-A(P1487C) that has a CPPSP motif and is indeed phosphorylated (Fig. 1B). Importantly, because the serine to alanine substitution in motif D (in LRP6 d)

causes significant decrease in LRP6 signaling (Fig. 5, A and B), motif D in full-length LRP6 is most likely phosphorylated. Therefore we have substantially extended our model that all five PPPSP motifs are phosphorylated by a common GSK3-dependent mechanism that is induced by Wnt stimulation.

It is worth noting that our unbiased alanine scanning mutagenesis has defined the core amino acid residues for motif A as PPPSPXS (Fig. 1). This matches perfectly with our previous study (14) and the consensus sequence of all five PPPSP motifs (Fig. 2A and supplemental Fig. S1). We previously proposed a “dual kinase” mechanism for PPPSPXS phosphorylation: Wnt induces GSK3-mediated PPPSP phosphorylation, which in turn primes the X phosphorylation by CKI; such dual phosphorylated PPPSPXS motif provides an optimal binding site for Axin (14). Given that our analysis has demonstrated common properties and GSK3-dependent phosphorylation for most and perhaps all PPPSP motifs, it seems likely that the dual kinase phosphorylation by GSK3-CKI applies to all of these motifs as well.

Our study uncovers an important feature of LRP6 signaling, i.e. the functional cooperation/synergy among five PPPSP motifs. Thus mutation of a single motif, in particular C, D, or E, results in a significant decrease in LRP6 function, whereas mutation of any two motifs in combination almost always causes a complete loss of LRP6 function (Fig. 5). The only exception is the mutation of A and B together (LRP6 ab), which has a milder effect but nonetheless leads to a significant loss of function (Fig. 5, C and D). This strong dependence of cooperativity may explain the conservation of five PPPSP motifs from Drosophila to human. The molecular mechanism underlying this cooperativity may have two aspects. The first is cooperativity in Axin binding. Although each individual PPPSP motif upon phosphorylation can bind to Axin (Fig. 3B), it seems unlikely that the five PPPSP motifs, which are clustered within 120 amino acid residues, are bound by five Axin molecules (of 800 amino acid residues each) simultaneously. Rather these five motifs likely increase the local concentration of binding sites for Axin to ensure tight LRP6-Axin association at the membrane. A future challenge will be to determine the stoichiometry of the LRP6-Axin complex. The second aspect is cooperativity in PPPSP phosphorylation. Our recent study suggests the existence of a local positive feedback loop between LRP6 and Axin; Axin not only binds to phosphorylated PPPSP motifs but also promotes and, in fact, is required for PPPSP phosphorylation via its recruitment of GSK3 (14). In such an amplification loop a phosphorylated PPPSP motif, by virtue of its binding to the Axin-GSK3 complex, promotes the phosphorylation of additional PPPSP motifs, and therefore its mutation will have major effects on the phosphorylation of other PPPSP motifs. Consistent with this notion, LRP6AN, which is constitutively activated and thus resembles Wnt stimulation, has much higher phosphorylation at motifs C and E than LRP6AN-C and LRP6AN-E, respectively (Fig. 4A), indicating that the existence of other functional PPPSP motifs has a profound stimulatory effect on C or E phosphorylation.

The dependence on the cooperativity among PPPSP motifs in the full-length LRP6 appears to “contrast” the results that a single PPPSP motif is sufficient to transfer signaling function to
LDLRΔN and to rescue the inactive LRP6ΔNm5 (6) (this study). One recent study suggests that LRP6ΔN has a propensity to aggregate (20). We also found that LDLRΔN-A forms large aggregates, and this aggregation is due to the LDLRΔN moiety and is independent of the transferred PPPSP motif. Such aggregates likely bring together multiple copies of PPPSP motifs in trans to form the amplification loop, thereby contrasting, but functionally equivalent to, the cooperativity of multiple PPPSP motifs in cis in the full-length LRP6. We note that it is curious as to why LRP6, in contrast to LRP6ΔN, shows strict dependence on multiplicity of PPPSP motifs in cis, which apparently cannot be substituted in trans if LRP6 forms Wnt-induced aggregates as suggested (20). In sum our results suggest that LRP6 function requires cooperativity of PPPSP motifs in promoting the phosphorylation of one another and in the recruitment of Axin, and this cooperativity likely represents a built-in amplifier that is essential for Wnt signaling amplification at the receptor level (7, 21). While this manuscript was in preparation, Wolf et al. (22) reported similar observations on PPPSP cooperation in LRP6 function.

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