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Journal of Cellular Biochemistry 108:886–895 (2009)

Journal of Cellular Biochemistry

Phosphorylation of PPP(S/T)P Motif of the Free LRP6 Intracellular Domain Is Not Required to Activate the Wnt/β-Catenin Pathway and Attenuate GSK3β Activity

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ABSTRACT

The canonical Wnt/β-catenin signaling pathway plays a critical role in numerous physiological and pathological processes. LRP6 is an essential co-receptor for Wnt/β-catenin signaling; as transduction of the Wnt signal is strongly dependent upon GSK3β-mediated phosphorylation of multiple PPP(S/T)P motifs within the membrane-anchored LRP6 intracellular domain. Previously, we showed that the free LRP6 intracellular domain (LRP6-ICD) can activate the Wnt/β-catenin pathway in a β-catenin and TCF/LEF-1 dependent manner, as well as interact with and attenuate GSK3β activity. However, it is unknown if the ability of LRP6-ICD to attenuate GSK3β activity and modulate activation of the Wnt/β-catenin pathway requires phosphorylation of the LRP6-ICD PPP(S/T)P motifs, in a manner similar to the membrane-anchored LRP6 intracellular domain. Here we provide evidence that the LRP6-ICD does not have to be phosphorylated at its PPP(S/T)P motif by GSK3β to stabilize endogenous cytosolic β-catenin resulting in activation of TCF/LEF-1 and the Wnt/β-catenin pathway. LRP6-ICD and a mutant in which all 5 PPP(S/T)P motifs were changed to PPP(A)P motifs equivalently interacted with and attenuated GSK3β activity in vitro, and both constructs inhibited the in situ GSK3β-mediated phosphorylation of β-catenin and tau to the same extent. These data indicate that the LRP6-ICD attenuates GSK3β activity similar to other GSK3β binding proteins, and is not a result of it being a GSK3β substrate. Our findings suggest the functional and regulatory mechanisms governing the free LRP6-ICD may be distinct from membrane-anchored LRP6, and that release of the LRP6-ICD may provide a complimentary signaling cascade capable of modulating Wnt-dependent gene expression. J. Cell. Biochem. 108: 886–895, 2009.

KEY WORDS: Wnt/β-CATENIN; LRP6; GSK3β; TCF/LEF-1

The canonical Wnt/β-catenin signaling pathway plays a critical role in numerous processes [He et al., 2004; Johnson and Rajamannan, 2006], including development of the central nervous system and neuronal plasticity [De Ferrari and Moon, 2006]. Further, alterations in the Wnt/β-catenin signaling pathway are implicated in numerous pathological conditions including various cancers and neurodegenerative disorders [Caricasole et al., 2004; He et al., 2004; De Ferrari and Moon, 2006; Johnson and Rajamannan, 2006; Scali et al., 2006].

A key component of the Wnt/β-catenin pathway is the regulation of the stability and abundance of cytosolic β-catenin, which acts as a nuclear co-activator for the T cell-specific transcription/lymphoid enhancer-binding factor 1 (TCF/LEF-1) family of transcription factors that mediate transcription of Wnt target genes [He et al., 2004; Tolwinski and Wieschaus, 2004a; MacDonald et al., 2008; Wolf et al., 2008]. Signaling activity of the Wnt/β-catenin pathway is mediated by the secreted lipid-modified glycoprotein Wnt and its interaction with the cell surface receptors Frizzled (Fz) and *L*ow density lipoprotein receptor *R*elated *P*rotein 6 (LRP6) (the closely related LRP5 can also act as a co-receptor for certain physiological processes) [He et al., 2004; Zeng et al., 2005, 2008; Bilic et al., 2007; MacDonald et al., 2008]. In the absence of the extracellular Wnt ligand, β-catenin as well as glycogen synthase kinase 3 beta (GSK3β), casein kinase 1 alpha (CKIα), and adenomatous polyposis coli (APC) are tethered in the cytosol to the scaffolding protein axin to form the multi-protein "destruction complex." In this complex, β-catenin is sequentially phosphorylated by CKIα at Ser45 and then by GSK3β at Thr41 and Ser33/37 [Liu et al., 2002]. Phosphorylation promotes β-catenin degradation via the ubiquitin-proteasome pathway resulting in low basal levels

Grant sponsor: NIH; Grant number: F31 AG030788.

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Received 27 February 2009; Accepted 22 July 2009 • DOI 10.1002/jcb.22318 • © 2009 Wiley-Liss, Inc.
Published online 26 August 2009 in Wiley InterScience (www.interscience.wiley.com).

of cytoplasmic β-catenin thus preventing β-catenin nuclear translocation and activation of TCF/LEF-1 [Liu et al., 2002; He et al., 2004; MacDonald et al., 2008; Wolf et al., 2008]. The classic view of Wnt/β-catenin pathway activation involves the Wnt ligand binding the extracellular domains of Fz and LRP6 thus inducing a Wnt-Fz-LRP6 ternary complex [Cong et al., 2004; He et al., 2004; Zeng et al., 2005, 2008; MacDonald et al., 2008]. The resulting complex facilitates/promotes phosphorylation of the membrane anchored LRP6 intracellular domain by GSK3β and CK1 [Zeng et al., 2005, 2008; Bilic et al., 2007]. Phosphorylation creates a binding site for axin thereby promoting recruitment of axin to the plasma membrane [Mao et al., 2001; Tamai et al., 2004; Zeng et al., 2005]. The resulting LRP6-axin association inhibits cytosolic β-catenin phosphorylation/degradation resulting in accumulation of β-catenin [He et al., 2004; Nusse, 2005; Zeng et al., 2008] and its subsequent translocation into the nucleus where it forms a complex with and converts TCF/LEF-1 into a transcriptional activator of Wnt target genes [Cong et al., 2004; He et al., 2004; Wolf et al., 2008]. Although these signaling processes are essential features of this pathway, numerous mechanistic, functional and regulatory aspects of the Wnt/β-catenin pathway however, still remain unclear [Cong et al., 2004; He et al., 2004; Tolwinski and Wieschaus, 2004b; Nusse, 2005; Gordon and Nusse, 2006; Wolf et al., 2008].

The intracellular domain of LRP6 contains a GSK3β phosphorylation motif, PPP(S/T)P, which is repeated five times. Full transduction of the Wnt signal requires and stimulates GSK3β-mediated phosphorylation of the PPP(S/T)P motifs in the membrane anchored LRP6 intracellular domain [Zeng et al., 2005; MacDonald et al., 2008; Wolf et al., 2008], as Wnt/β-catenin pathway activation is abolished when all five PPP(S/T)P motifs been deleted or each Ser/Thr is converted to an Ala [He et al., 2004; Tamai et al., 2004; Zeng et al., 2005; MacDonald et al., 2008; Wolf et al., 2008]. Although several models have been proposed, the regulatory and functional mechanism(s) governing LRP6 phosphorylation have not been fully elucidated and certain aspects remain controversial [Liu et al., 2003; Cong et al., 2004; He et al., 2004; Zeng et al., 2005, 2008; Bilic et al., 2007; Cselenyi et al., 2008; Wolf et al., 2008]. Data does suggest the LRP6-axin interaction promotes axin degradation to stabilize cytosolic β-catenin [He et al., 2004; Kofron et al., 2007]. However, there is also data to suggest a more immediate Wnt-mediated, unidentified cooperative secondary mechanism exists for affecting cytosolic β-catenin [Willert et al., 1999; Hino et al., 2005; Liu et al., 2005; Mi et al., 2006; Cselenyi et al., 2008].

GSK3β is a dynamic Ser/Thr kinase that phosphorylates numerous substrates, including more than a dozen transcription factors [Jope and Johnson, 2004]. Regulation of GSK3β is a complex process which must be tailored for each substrate to avoid indiscriminate phosphorylation and is achieved by a combination of phosphorylation, subcellular distribution and protein complex formation mediated by GSK3β-binding proteins [Jope and Johnson, 2004]. This diverse regulatory scheme allows GSK3β to act as a positive or negative regulator of the Wnt/β-catenin pathway (depending upon its subcellular localization) [Zeng et al., 2005, 2008]. As a negative regulator, cytosolic GSK3β (and GSK3α) phosphorylates β-catenin to promote its degradation, phosphorylates APC to increase its binding to β-catenin [Hart et al., 1998] and phosphorylates axin in the destruction complex which stabilizes axin and increases its affinity for β-catenin [Yamamoto et al., 1999]. GSK3β can also act as a positive modulator through Wnt stimulated phosphorylation of the membrane anchored LRP6 intracellular domains PPP(S/T)P motifs [Zeng et al., 2005, 2008; MacDonald et al., 2008]. Importantly, it has been shown the Wnt/β-catenin pathway, in an LRP6 dependent manner, can regulate GSK3β-mediated phosphorylation of proteins implicated in Alzheimer disease, such as tau [Caricasole et al., 2004; Scali et al., 2006].

Our group has previously shown that exogenous expression of the free (i.e., not anchored to the membrane) LRP6 intracellular domain (LRP6-ICD) attenuates cytosolic β-catenin phosphorylation/degradation resulting in constitutive activation of TCF/LEF-1 and the Wnt/β-catenin pathway [Mi and Johnson, 2005]. Moreover, the LRP6-ICD can directly interact with and attenuate GSK3β activity including GSK3β-mediated phosphorylation of β-catenin [Mi et al., 2006; Cselenyi et al., 2008] and tau [Mi et al., 2006]. Previously our group has shown in situ that exogenous LRP6 undergoes regulated intramembranous proteolysis (RIP) resulting in the formation and release of an LRP6-ICD [Mi and Johnson, 2007]. Taken as a whole, these data strongly indicate that the LRP6-ICD is a biologically functional protein capable of cooperatively interacting in the Wnt/β-catenin pathway [Mi and Johnson, 2005; Mi et al., 2006; Cselenyi et al., 2008], as well as influencing general GSK3β activity [Mi et al., 2006]. However, it is not clear if the LRP6-ICD PPP(S/T)P motifs must be phosphorylated by GSK3β to attenuate GSK3β activity, as well as function in the Wnt/β-catenin pathway, similar to the membrane anchored LRP6 intracellular domain, which is the focus of this study.

MATERIALS AND METHODS

DNA CONSTRUCTS

All LRP6-ICD constructs (including LRP6-ICDx5m) consist only of the intracellular domain of full length human LRP6, and do not localize to the membrane [Mi and Johnson, 2005]. The LRP6-ICD constructs were cloned from full length human LRP6 cDNA (NM-002336, nucleotides 4326–4919, residue 1416–1613 of LRP6) [Mi and Johnson, 2005]. Wild-type LRP6-ICD contains all five of the GSK3β phosphorylation motifs, PPP(S/T)P, and is phosphorylated by GSK3β in vitro [Mi et al., 2006]. For LRP6-ICDx5m constructs, the Ser or Thr in all five PPP(S/T)P motifs were mutated to Ala and this construct is not phosphorylated by GSK3β in vitro [Mi et al., 2006]. Wild-type LRP6-ICD-HA and GFP-LRP6-ICD, GSK3β-HA, tau and His-p53 constructs were described previously [Zhang et al., 2002; Mi and Johnson, 2005; Mi et al., 2006]. To make the LRP6-ICDx5m-HA and GFP-LRP6-ICDx5m, GST-LRP6-ICDx5m [Mi et al., 2006] was used as a template for PCR amplification. The following PCR primers, digestion, and vectors were used for each: forward (5′-GGA-ATT-CGC-CAT-GGG-ACC-AGC-TTC-TGT-GCC-TCT-3′) and reverse (5′-GGA-ATT-CGG-AGG-AGT-CTG-TAC-AGG-GAG-CGG-GTG-GCG-GTG-3′), digested with and subcloned into the *Eco*R1 site of the same vector as GSK3β-HA (LRP6-ICDx5m-HA); forward (5′-CCG-GAA-TTC-GGG-ACC-AGC-TTC-TGT-GCC-TCT-TGG-TTA-TGT-3′) and reverse (5′-GTG-GAT-CCT-TAG-GAG-GAG-TCT-GTA-

CAG-GGA-GCG-GGT-GGC-GGT-GGG-3′), digested with and subcloned into the *Eco*R1 and *Bam*H1 site of the pEGFP-C1 (BD Biosciences) (GFP-LRP6-ICDx5m). The integrity of all constructs was confirmed by sequence analysis.

CELL CULTURE AND CONDITIONED MEDIA AND TRANSIENT TRANSFECTIONS

CHO, HEK 293T, COS-7, L-cells, and L-Wnt3a cell lines were maintained according to the supplier's protocols (ATCC). Conditioned media collected from L-cells provided non-Wnt3a control conditioned media (LCM) while media from L-Wnt3a cells provided Wnt3a conditioned media (WCM). Conditioned media was prepared following the protocol provided by ATCC. All transient transfections were done using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's protocol. The final amount of cDNA transfected into the cells was always equalized by using a control β-galactosidase (LacZ) construct.

TCF/LEF-1 TOPFLASH REPORTER ASSAY

COS-7 cells were transfected with 0.2 μg Topflash-luciferase reporter (Upstate), 0.2 μg LEF-1 (from Dr. R Grosschedl) and 0.005 μg of TK-*Renilla* (Promega). Additionally, cells were transfected with His-p53, wild-type LRP6-ICD-HA, LRP6-ICDx5m-HA or a control LacZ construct. Seven hours post-transfection, media was changed to WCM or LCM and cells were collected in luciferase assay lysis buffer (Promega) 48 h post-transfection. Luciferase activity was measured using Promega Dual-Luciferase reporter assay kit with a luminometer (Turner Designs). All experiments were performed at least three times and each time the measurements were done in triplicate. COS-7 cells were chosen as the cell model for the reporter assay to facilitate comparisons with our previously published findings [Mi and Johnson, 2005].

IMMUNOBLOTTING AND ANTIBODIES

Unless indicated, 48 h post-transfection cells were collected in ice cold lysis buffer containing 2% sodium dodecyl sulfate (SDS), 250 mM Tris-Cl (pH 7.4), 10% glycerol, 5 mM EDTA and 5 mM EGTA with phosphatase and protease inhibitors; electrophoresed on 10% SDS–polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted as previously described [Mi et al., 2006]. Determination of endogenous total β-catenin, phospho-β-catenin, and cytosolic β-catenin levels were carried out using non-detergent lysis buffer as previously described [Mi and Johnson, 2005; MacDonald et al., 2008]. The following antibodies were used: polyclonal anti-phospho-β-catenin (Ser 33/37, Thr 41, Cell Signaling), monoclonal anti-β-catenin (BD Biosciences), monoclonal PHF-1 (anti-tau Ser396/404, a gift from Dr. P. Davies [Hong et al., 1997]), monoclonal Tau5 (anti-total tau, a gift from Dr. L. Binder [Mi et al., 2006]), monoclonal anti-GSK3β (BD Biosciences), polyclonal anti-phospho GSK3β (Ser9, Cell Signaling), monoclonal anti-HA (Covance), monoclonal anti-GFP (Roche Applied Science), monoclonal anti-α-tubulin (Sigma) and polyclonal anti-phospho LRP6 (Ser1490, Cell Signaling). Densitometric quantitation of tau phosphorylation at PHF-1 epitope (Ser396/404) was carried out by normalizing the values to total tau levels and expressing the data as a percent of control.

IMMUNOPRECIPITATION

CHO cells were co-transfected with GSK3β-HA along with pEGFP-C1 empty vector (eGFP), wild-type GFP-LRP6-ICD, or GFP-LRP6-ICDx5m. Forty-eight hours post-transfection, cells were collected in ice cold immunoprecipitation lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris-Cl pH 7.4, 1 mM EGTA and 1 mM EDTA with protease and phosphatase inhibitors) and immunoprecipitation was performed as previously described [Mi et al., 2006].

GSK3 ACTIVITY ASSAY

HEK 293T cells were co-transfected with GSK3β-HA along with eGFP (empty vector), wild-type GFP-LRP6-ICD or GFP-LRP6-ICDx5m. Forty-eight hours post-transfection, cells were collected with ice cold immunoprecipitation lysis buffer and the lysates were immunoprecipitated using 1.5 μg anti-HA antibody (to directly IP GSK3β-HA) or 1 μg of the anti-GFP antibody (to immunoprecipitate wild-type GFP-LRP6-ICD or GFP-LRP6-ICDx5m and the associated GSK3β). Immunoprecipitates were washed two times with immunoprecipitation lysis buffer and two times with GSK3β kinase wash buffer (20 mM Tris-Cl, pH 7.5, 5 mM MgCl2, and 1 mM dithiothreitol). The immunoprecipitates were then used for an in vitro GSK3β activity assay using phosphoglycogen synthase peptide 2 as a substrate as previously described [Stoothoff et al., 2005]. GSK3β-HA activity were normalized to GSK3β-HA levels in the sample and the data were expressed as a percent of control (GSK3β-HA alone) [Stoothoff et al., 2005]. HEK 293T cells were used in the assay as they were the model system we used previously [Stoothoff et al., 2005] and because they are a commonly used cell model for these types of studies [MacDonald et al., 2008; Wolf et al., 2008; Zeng et al., 2008].

DATA ANALYSIS

Data were analyzed using ANOVA between individual groups and considered significantly different when $P < 0.05$. Results were expressed as mean ± SE.

RESULTS

LRP6-ICD IS PHOSPHORYLATED BY GSK3β IN SITU

It has been shown in vitro that GSK3β phosphorylates wild-type LRP6-ICD but not LRP6-ICDx5m (Ser/Thr in the five PPP(S/T)P motifs mutated to Ala) [Mi et al., 2006]. Similar results were obtained in situ as wild-type LRP6-ICD but not LRP6-ICDx5m was phosphorylated at Ser1490 (i.e., the first PPP(S/T)P motif of LRP6-ICD) (Fig. 1A). Phosphorylation of the wild-type LRP6-ICD at Ser1490 was inhibited in the presence of the GSK3β inhibitor, LiCl (Fig. 1A). Neither expression of wild-type LRP6-ICD nor LRP6-ICDx5m altered inhibitory phosphorylation of GSK3β (see Fig. 6A; pSer9-GSK3β). These data clearly demonstrate that wild-type LRP6-ICD can be phosphorylated at its PPP(S/T)P motif by endogenous GSK3β in situ.

PHOSPHORYLATION OF ENDOGENOUS, MEMBRANE-ANCHORED LRP6 IS NOT ALTERED BY LRP6-ICD

Wnt stimulated phosphorylation of the membrane anchored LRP6 PPP(S/T)P motif by GSK3β is required for activation of the

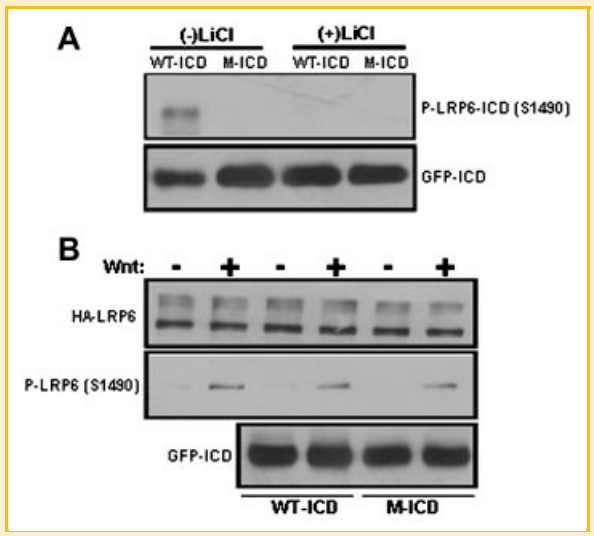

Fig. 1. LRP6-ICD is phosphorylated at its PPP(S/T)P motif in situ by GSK3β and does not effect phosphorylation of membrane anchored LRP6. A: HEK 293T cells were transfected with wild-type GFP-LRP6-ICD (WT-ICD) or GFP-LRP6-ICDx5m (M-ICD) in the presence or absence of 50 mM LiCl. WT-ICD (lane 1) but not M-ICD (lane 2) is phosphorylated at the Ser1490 PPP(S/T)P motif. Inhibition of WT-ICD phosphorylation (lane 3) by LiCL (selective GSK3β inhibitor) confirms GSK3β can phosphorylate WT-ICD in situ. B: HEK 293T cells were co-transfected with HA-LRP6 along with eGFP (empty vector), wild-type GFP-LRP6-ICD (WT-ICD) or GFP-LRP6-ICDx5m (M-ICD). Cells were treated with non-Wnt3a media (lane 1, 3, and 5) or Wnt3a media (lane 2, 4, and 6). eGFP transfected samples were equal (data not shown). Phosphorylation of full length, membrane anchored LRP6 at the Ser1490 PPP(S/T)P motif was not affected by WT-ICD or M-ICD expression.

Wnt/β-catenin pathway [Zeng et al., 2005, 2008; MacDonald et al., 2008; Wolf et al., 2008]. However it is not known if LRP6-ICD effects GSK3β-mediated phosphorylation of full length LRP6 under basal and/or Wnt stimulated conditions. Figure 1B shows that over-expression of wild-type LRP6-ICD or LRP6-ICDx5m does not alter phosphorylation of LRP6 at Ser 1490 in the absence or presence of Wnt-3A. Therefore, despite its ability to interact with and attenuate GSK3β activity, the data indicate expression of cytosolic LRP6-ICD does not affect the membrane localized [Zeng et al., 2008] pool of GSK3β responsible for LRP6 phosphorylation.

LRP6-ICD CONSTITUTIVELY ACTIVATES AND AMPLIFIES Wnt3A-INDUCED TCF/LEF-1 ACTIVATION INDEPENDENT OF PPP(S/T)P MOTIF PHOSPHORYLATION

Activation of TCF/LEF-1 is a hallmark of Wnt/β-catenin pathway activation and the TCF/LEF-1 TOPFLASH reporter is commonly used to measure activation of this pathway [Cong et al., 2004; Mi and Johnson, 2005; Zeng et al., 2005, 2008; MacDonald et al., 2008; Wolf et al., 2008]. We previously showed that wild-type LRP6-ICD can activate TCF/LEF-1 independent of Wnt stimulation, as well as amplify Wnt-mediated activation of TCF/LEF-1 [Mi and Johnson, 2005]. In Figure 2 we show the LRP6-ICDx5m also activates TCF/LEF-1 in the absence of exogenously added Wnt. Further, LRP6-ICDx5m significantly increased Wnt3a-induced TCF/LEF-1 activity compared with Wnt3a treated control (LacZ) cells (Fig. 2).

PHOSPHORYLATION OF LRP6-ICD PPP(S/T)P BY GSK3β IS NOT REQUIRED FOR LRP6-ICD MEDIATED STABILIZATION OF CYTOPLASMIC β-CATENIN

In the absence of the Wnt ligand, GSK3β phosphorylates cytosolic β-catenin at Thr 41 and Ser 33/37 in the destruction complex priming β-catenin for ubiquitinylation and degradation [Liu et al., 2002]. Activation of the Wnt/β-catenin pathway results in stabilization of cytosolic β-catenin which is characterized by a decrease in phospho-β-catenin and a concomitant increase in cytosolic β-catenin [Liu et al., 2002; He et al., 2004; Mi and Johnson, 2005; Zeng et al., 2005, 2008; MacDonald et al., 2008; Wolf et al., 2008]. We previously demonstrated that in the absence of exogenous Wnt treatment, expression of exogenous wild-type LRP6-ICD promotes stabilization of endogenous cytosolic β-catenin [Mi and Johnson, 2005]. Similar to wild-type LRP6-ICD, LRP6-ICDx5m also attenuated endogenous GSK3β-mediated phosphorylation of endogenous cytosolic β-catenin (Fig. 3A). Attenuation of endogenous cytosolic β-catenin phosphorylation by LRP6-ICDx5m occurred concurrently with an accumulation of endogenous cytosolic β-catenin (Fig. 3B). Thus, the data shows that LRP6-ICD does not have to be phosphorylated by GSK3β at its PPP(S/T)P motif to attenuate endogenous GSK3β-mediated phosphorylation and subsequent stabilization of endogenous cytosolic β-catenin. Stabilization of cytosolic β-catenin is specific to LRP6-ICD as expression of another GSK3β modulatory protein, p53 [Watcharasit et al., 2003], did not result in cytosolic β-catenin stabilization (Fig. 3C).

INTERACTION BETWEEN LRP6-ICD AND GSK3β DOES NOT REQUIRE PHOSPHORYLATION OF THE LRP6-ICD PPP(S/T)P MOTIF

GSK3β is a dynamic kinase whose activity is tightly regulated in part by GSK3β binding proteins, such as FRAT-1 and p53 [Thomas et al., 1999; Culbert et al., 2001; Watcharasit et al., 2002]. Wild-type LRP6-ICD interacts with GSK3β in vitro and in situ [Mi et al., 2006]. GSK3β can also phosphorylate wild-type LRP6-ICD, but not LRP6-ICDx5m at the PPP(S/T)P motif, in vitro [Mi et al., 2006] and in situ (Fig. 1A). However, it is unknown if phosphorylation of the PPP(S/T)P motif in LRP6-ICD is required for LRP6-ICD to interact with GSK3β. To test this, a co-immunoprecipitation assay was carried out. These data demonstrate that LRP6-ICDx5m interacts with exogenous GSK3β in a manner similar to wild-type LRP6-ICD (Fig. 4). Wild-type LRP6-ICD (In accordance with previous findings [Mi et al., 2006]), and LRP6-ICDx5m also apparently interacted with endogenous GSK3β (Fig. 4C). Therefore, the data suggest the LRP6-ICD can act as a GSK3β binding protein independent of being a GSK3β substrate.

PHOSPHORYLATION OF THE LRP6-ICD PPP(S/T)P MOTIF IS NOT REQUIRED TO INHIBIT GSK3β ACTIVITY IN VITRO

Previously our group showed that wild-type LRP6-ICD inhibits GSK3β activity towards a primed peptide substrate [Mi et al., 2006].

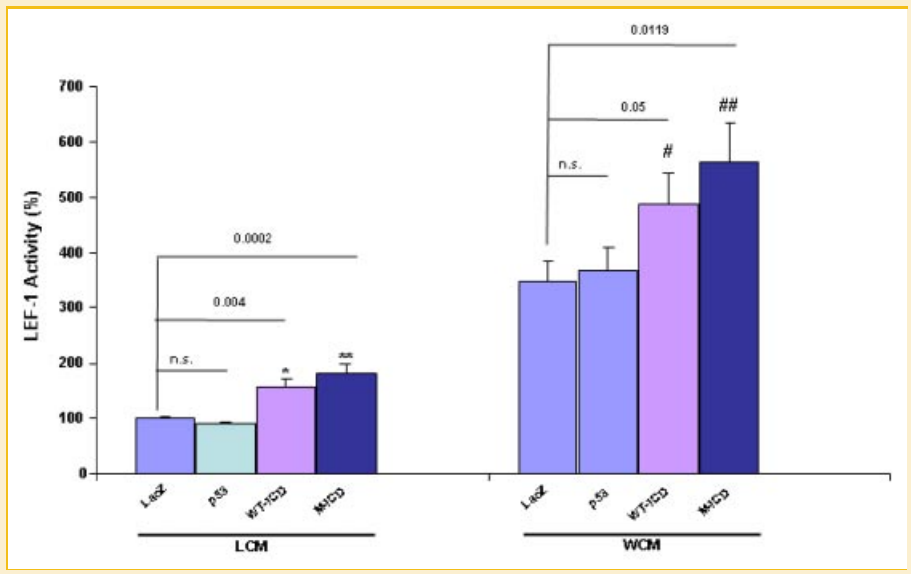

Fig. 2. LRP6-ICDx5m constitutively activates and potentiates Wnt3a-induced TCF/LEF-1 activation. Cos-7 cells were transfected with luciferase assay reporter constructs along with LacZ (control), his-p53, LRP6-ICD-HA (WT-ICD) or LRP6-ICDx5m-HA (M-ICD). Seven hours post-transfection, Cells were treated with non-Wnt3a media (LCM) or Wnt3a media (WCM) and TCF/LEF-1 activity was measured 48 h post-transfection. Expression of WT-ICD and M-ICD were equal as determined by immunoblotting (data not shown). LCM treated cells transfected with WT-ICD or M-ICD compared to LacZ ($^{*}P = 0.004$, $^{**}P = 0.0002$). WCM treated cells transfected with WT-ICD or M-ICD compared to LacZ ($^{\#}P = 0.05$, $^{\#\#}P = 0.0119$). Expression of the GSK3β effector protein, p53, did not activate TCF/LEF-1 compared to LacZ.[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

However, it is unclear if attenuation of GSK3β activity was a result of LRP6-ICD acting as a competitive phospho-acceptor, thereby attenuating the phosphorylation of other GSK3β substrates, or whether it was acting as an inhibitor independent of its phosphorylation by GSK3β similar to other GSK3β binding proteins (e.g., FRAT-1) [Thomas et al., 1999; Culbert et al., 2001]. Therefore an in vitro GSK3β activity assay was carried out using exogenous GSK3β immunoprecipitated directly (GSK3β alone) or co-immunoprecipitated with wild-type LRP6-ICD (GFP-LRP6-ICD/GSK3β-HA) or LRP6-ICDx5m (GFP-LRP6-ICDx5m/GSK3β-HA). Figure 5

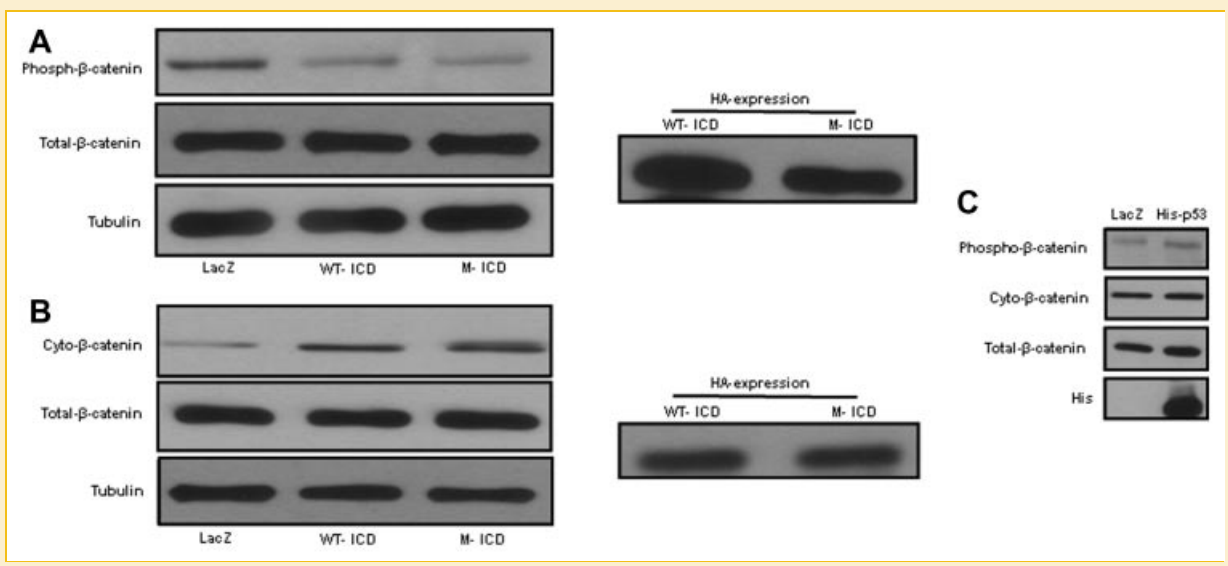

Fig. 3. Phosphorylation of LRP6-ICD PPP(S/T)P by GSK3β is not required for LRP6-ICD mediated stabilization of cytoplasmic β-catenin. HEK 293T cells were transfected with LacZ (control), LRP6-ICD-HA (WT-ICD) or LRP6-ICDx5m-HA (M-ICD). Twenty-four hours post-transfection, cells were collected and cytosolic fractions were probed for (A) endogenous cytosolic phospho-β-catenin (Ser33/37/Thr41) or (B) total endogenous cytosolic β-catenin. Total cellular β-catenin levels did not change. Insert labeled HA-expression shows equal expression between WT-ICD (band 1) and M-ICD (band 2) for both (A,B). C: HEK 293T cells were transfected with LacZ or His-p53, collected and fractionated as described above. Data shows overexpression of the GSK3β effector protein, p53, does not result in stabilization of cytoplasmic β-catenin.

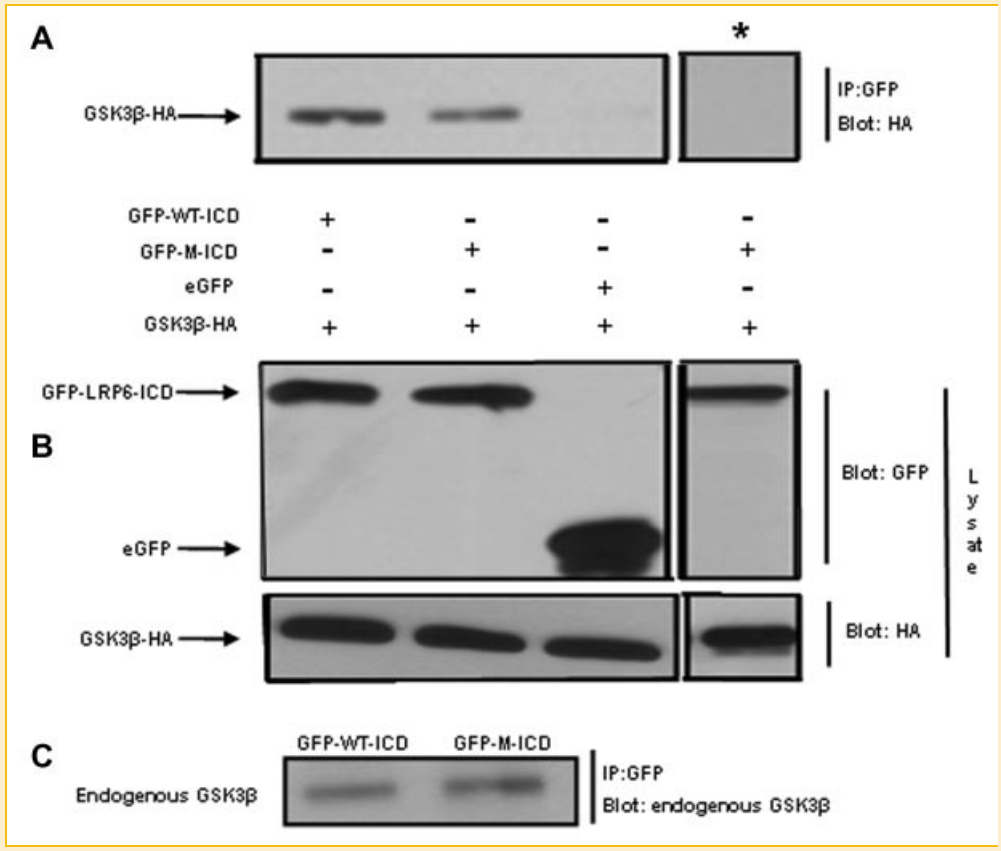

Fig. 4. Interaction between LRP6-ICD and GSK3β does not require phosphorylation of the PPP(S/T)P motif. CHO cells were co-transfected with wild-type GFP-LRP6-ICD (WT-ICD), GFP-LRP6-ICDx5m (M-ICD) or eGFP-C1 (empty vector) along with GSK3β-HA. A: Cell lysates were immunoprecipitated with anti-GFP antibody and probed with anti-HA antibody then stripped and reprobed with anti-GFP antibody. Immunoprecipitation of the GFP constructs was equal (data not shown). B: Represents expression of indicated constructs for each sample. C: Shows GFP-LRP6-ICD (GFP-WT-ICD) and GFP-LRP6-ICDx5m (GFP-M-ICD) immunoprecipitate with endogenous GSK3β. Note (*) refers to control lysate that was incubated with non-immune mouse IgG1 antibody to prevent non-specific binding.

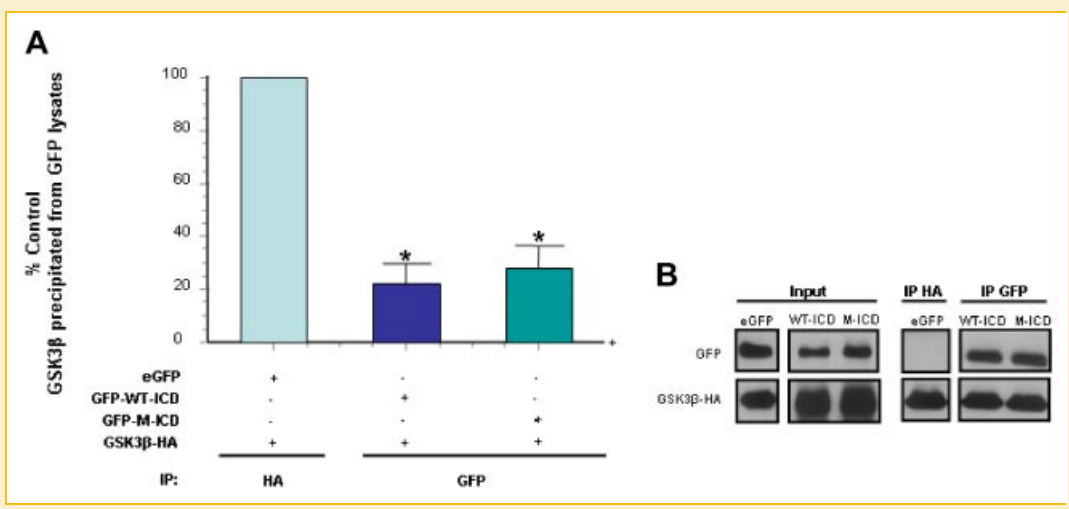

Fig. 5. Phosphorylation of the LRP6-ICD PPP(S/T)P motif is not required to inhibit GSK3β-mediated phosphorylation of a primed peptide in vitro. HEK 293T were transfected with the constructs indicated and immunoprecipitated with either the anti-HA antibody to directly precipitate exogenous GSK3β-HA or with the anti-GFP antibody to immunoprecipitate wild-type GFP-LRP6-ICD (GFP-WT-ICD) or GFP-LRP6-ICDx5m (GFP-M-ICD). The immunoprecipitates were then used in kinase assays. A: When GFP-WT-ICD or GFP-M-ICD was immunoprecipitated and therefore all the GSK3β-HA in the assay was bound to GFP-WT-ICD or GFP-M-ICD, GSK3β-HA phosphorylation of the primed peptide was significantly decreased compared to when only GSK3β-HA was present. Data were normalized to immunoprecipitated GSK3β levels and expressed as percent of values obtained with GSK3β-HA alone (immunoprecipitated with anti-HA antibody). Data are presented as mean ± SE of seven experiments. $^{*}P < 0.001$ compared to GSK3β-HA alone. B: Representative immunoblots showing levels of transfected proteins (input) and immunoprecipitated (IP) GSK3β-HA from cell lysates used for the GSK3β assay. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

shows that both wild-type LRP6-ICD and LRP6-ICDx5m significantly attenuated immunoprecipitated GSK3β activity towards a primed peptide substrate by ~75%. These data indicate that LRP6-ICD attenuation of GSK3β activity, similar to other GSK3β binding proteins [Thomas et al., 1999; Culbert et al., 2001], is not a result of it being a GSK3β substrate/phosphate acceptor.

IN SITU INHIBITION OF GSK3β-MEDIATED TAU PHOSPHORYLATION BY LRP6-ICD DOES NOT REQUIRE PHOSPHORYLATION OF THE LRP6-ICD PPP(S/T)P MOTIF

Tau is a neuronal microtubule-associated protein tightly regulated by site-specific phosphorylation. GSK3β is a predominant tau kinase capable of phosphorylating numerous epitopes on tau [Stoothoff and Johnson, 2005]. Wild-type LRP6-ICD attenuated endogenous GSK3β-mediated phosphorylation of substrates including tau [Mi et al., 2006]. Figure 6 shows endogenous GSK3β readily phosphorylates exogenous tau at Ser396/404, as it is inhibited by LiCl (eGFP; (+) LiCl), a selective GSK3 inhibitor [Hong et al., 1997]. Compared to the control sample (eGFP; (−) LiCl) both wild-type LRP6-ICD and LRP6-ICDx5m significantly attenuated phosphorylation of exogenous tau at Ser396/404 by endogenous GSK3β by ~25%. In agreement with the in vitro (Fig. 5) and in situ phospho-β-catenin data (Fig. 3A), these data show the LRP6-ICD can act as a general GSK3β modulatory protein independent of PPP(S/T)P phosphorylation by GSK3β.

DISCUSSION

Over the past few years, our knowledge and understanding of the Wnt/β-catenin pathway has increased significantly. Regulation of cytosolic β-catenin in the absence of Wnt signaling has been well characterized. However, there are more questions than answers concerning activation of the pathway, as well as stabilization of cytosolic β-catenin including: the identity of all the proteins that actually participate in the pathway, the specific localization of these proteins (i.e., membrane, cytosol, nuclear), the exact role that the proteins play in the pathway, and what posttranslational events play a role in propagating the Wnt signal [Cong et al., 2004; He et al., 2004; Tolwinski and Wieschaus, 2004b; Nusse, 2005; Gordon and Nusse, 2006; Cselenyi et al., 2008; Wolf et al., 2008].

Phosphorylation of the membrane anchored LRP6 intracellular domain PPP(S/T)P motif by GSK3β (as well as GSK3α [MacDonald et al., 2008]) plays a significant role in LRP6-mediated activation of the Wnt/β-catenin pathway [Zeng et al., 2005, 2008; MacDonald et al., 2008; Wolf et al., 2008]. Data indicate that phosphorylation of the PPP(S/T)P motif creates a binding site for axin to form the membrane anchored LRP6-axin complex resulting in stabilization of cytosolic β-catenin [Mao et al., 2001; He et al., 2004; Tamai et al., 2004; Nusse, 2005; Zeng et al., 2005; MacDonald et al., 2008]. However, the mechanism(s) by which phosphorylated, membrane bound LRP6 can interact with and recruit cytosolic axin to the plasma membrane as well as alter the axin-mediated destruction complex function has not been completely defined and/or agreed upon [Cong et al., 2004; He et al., 2004; Nusse, 2005; Chen et al., 2006; Bilic et al., 2007; Zeng et al., 2008]. Data suggest the LRP6/5-axin interaction stimulates axin degradation [Mao et al., 2001; Tolwinski et al., 2003; Kofron et al., 2007] and this has been proposed to play a critical role in the activation of the Wnt/β-catenin signaling pathway [Tolwinski and Wieschaus, 2004b]. However, Wnt-mediated stabilization of β-catenin in cultured

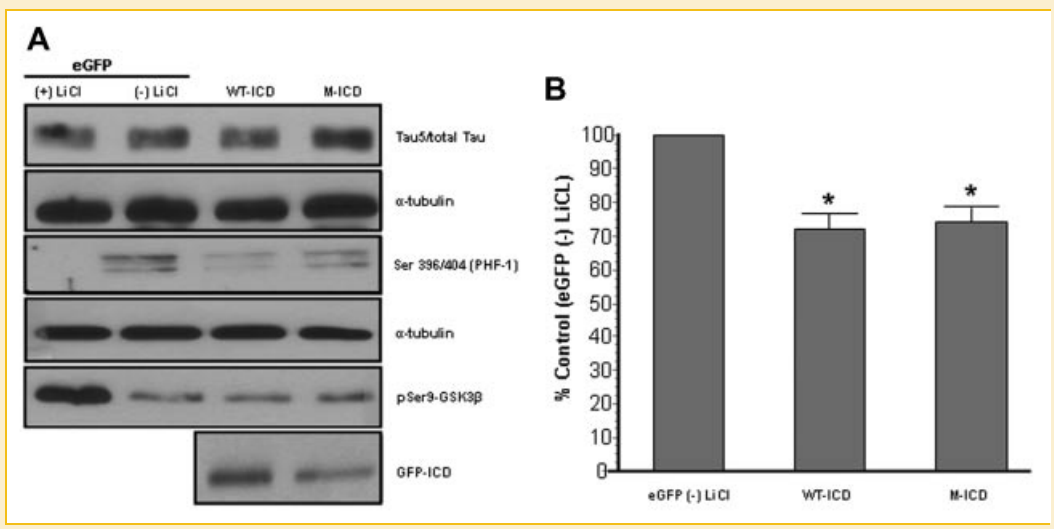

Fig. 6. Inhibition of GSK3β mediated Tau phosphorylation by LRP6-ICD does not require phosphorylation of the LRP6-ICD PPP(S/T)P motif. CHO cells were co-transfected with eGFP (empty vector) +50 mM LiCl (GSK3 inhibitor), eGFP (empty vector) no LiCl treatment, wild-type GFP-LRP6-ICD (WT-ICD) or GFP-LRP6-ICDx5m (M-ICD) along with exogenous tau. A: Representative immunoblots showing total tau (Tau5) and tau phosphorylated at the unprimed pSer396/404 (PHF-1) site. Expression levels of transfected WT-ICD and M-ICD are shown (GFP). eGFP transfected samples were equal (data not shown). α-tubulin was used as the loading control for Tau5 and PHF-1 blots. Phosphorylation state of tau for eGFP transfected cells (lane 1 and 2) show endogenous GSK3β is sufficient to phosphorylate exogenous tau at the PHF-1 site. pSer9-GSK3β levels are not altered by WT-ICD or M-ICD. B: Quantitation of immunoblot data for PHF-1 epitopes. PHF-1 immunoreactivity was normalized to total tau levels in each sample and expressed as percent of control (eGFP (−) LiCl). Data for LiCl treated cells are not shown in the graph. Data are presented as mean ± SE of three separate experiments. *$P < 0.05$ for WT-ICD (lane 2) and M-ICD (lane 3) compared to eGFP (−) LiCl treatment (lane 2).

mammalian cells occurs several hours before any detectable axin degradation [Willert et al., 1999; Hino et al., 2005; Liu et al., 2005]. Therefore, the data suggest an unknown, cooperative secondary mechanism (not alternative/mutually exclusive) may exists for modulating activation of the Wnt/β-catenin pathway [Tolwinski and Wieschaus, 2004b].

Our group has recently shown that exogenous LRP6 undergoes intramembranous proteolytic cleavage resulting in release of the LRP6 intracellular domain from the membrane (LRP6-ICD) [Mi and Johnson, 2007]. This type of processing has also been demonstrated for other LRP6 related family members, such as LRP1 [May et al., 2002; Kinoshita et al., 2003; Zou et al., 2004; Hoe and Rebeck, 2005]. It has also been shown in the absence of exogenous Wnt treatment, exogenous expression of the free LRP6-ICD results in activation of the Wnt/β-catenin pathway via cytosolic β-catenin stabilization [Mi and Johnson, 2005; Cselenyi et al., 2008] and TCF/LEF-1 activation [Mi and Johnson, 2005]. Although data indicate the LRP6-ICD is a biologically functional protein capable of cooperatively interacting in the Wnt/β-catenin pathway, its regulatory and functional mechanism(s) have not been elucidated. In vitro [Mi et al., 2006] and in situ (Fig. 1A) data show that the LRP6-ICD's PPP(S/T)P motifs can be phosphorylated by GSK3β. However, it is unknown if LRP6-ICD function is dependent upon phosphorylation of its PPP(S/T)P motifs by GSK3β similar to the membrane anchored LRP6 intracellular domain [Zeng et al., 2005, 2008; MacDonald et al., 2008; Wolf et al., 2008].

Here we show that in contrast to the membrane anchored LRP6 intracellular domain, the free LRP6-ICD does not require phosphorylation of its PPP(S/T)P motif by GSK3β in order to stabilize cytosolic β-catenin. Results also demonstrate the LRP6-ICD can activate TCF/LEF-1 in the absence of, as well as amplify Wnt-mediated TCF/LEF-1 activation. The data suggest LRP6-ICD is capable of functioning in the Wnt/β-catenin pathway independent of GSK3β-mediated phosphorylation of its PPP(S/T)P motifs. One explanation is that in contrast to membrane anchored LRP6 intracellular domain, the LRP6-ICD may function in the Wnt/β-catenin pathway without having to interact with axin. The ability of LRP6-ICD to functionally affect GSK3β-mediated phosphorylation of β-catenin independent of axin has recently been demonstrated in vitro [Mi et al., 2006; Cselenyi et al., 2008]. Therefore phosphorylation of the axin binding PPP(S/T)P motifs in the LRP6-ICD may not be required for the LRP6-ICD to function in the Wnt/β-catenin pathway. Activation of the pathway was specific to LRP6-ICD as expression of another GSK3β modulatory protein, p53 [Watcharasit et al., 2003], did not result in TCF/LEF-1 activation (Fig. 2) and/or cytosolic β-catenin stabilization (Fig. 3C).

While significant, LRP6-ICD-mediated stabilization of cytosolic β-catenin and activation of TCF/LEF-1 in the absence of exogenous Wnt treatment is less than would be expected for the primary activator of the pathway. However, current (Fig. 2) and previous data [Mi and Johnson, 2005] shows the LRP6-ICD is also capable of significantly amplifying Wnt-mediated activation of the signaling pathway (ex. TCF/LEF-1 activation). Taken together, data suggest that the LRP6-ICD can cooperatively interact in the Wnt/β-catenin pathway to amplify/modulate the Wnt signal. Indeed, such cooperativity/amplification models have been proposed for recently identified pathway components such as R-Spondin [Binnerts et al., 2007].

The LRP6-ICD has also been shown to directly interact with and attenuate GSK3β activity including phosphorylation of GSK3β substrates such as β-catenin [Mi et al., 2006; Cselenyi et al., 2008] and tau [Mi et al., 2006] in an axin independent manner. However, it was uncertain if interaction and attenuation is conditional upon LRP6-ICD being a substrate for GSK3β. Here we show the LRP6-ICD/GSK3β interaction is phosphorylation independent and not a result of the LRP6-ICD acting as a substrate for GSK3β phosphorylation. Similar interaction/association has also been shown for other GSK3β binding/interacting/effector proteins such as p53 [Watcharasit et al., 2003]. We also show that LRP6-ICD does not have to be phosphorylated by GSK3β to attenuate GSK3β-mediated phosphorylation of a primed peptide in vitro (Fig. 5) as well as in situ inhibition of two prominent GSK3β substrates, β-catenin (Fig. 3), and tau (Fig. 6). The data suggest the LRP6-ICD functions in a manner similar to other GSK3β binding proteins, such as FRAT-1, in its ability to attenuate GSK3β in a non-substrate/phosphorylation dependent manner [Thomas et al., 1999; Culbert et al., 2001].

A recent study by Cselenyi et al. [2008] presents findings that seemingly contrast with the current data. In this study it was suggested that LRP6-ICD requires GSK3β-mediated phosphorylation of its PPP(S/T)P motifs to inhibit β-catenin phosphorylation by GSK3β. The study also suggested the LRP6-ICD does not act as a general GSK3β effector protein but is only capable of attenuating GSK3β-mediated phosphorylation of β-catenin independent of axin degradation. Such conflicting data may be due to the fact that Cselenyi et al. used a *Xenopus* egg extract/in vitro system as opposed to a mammalian cell culture/in situ based system utilized in the current study. Conflicting data concerning Wnt/β-catenin pathway components is not uncommon in the Wnt field [Cong et al., 2004; He et al., 2004; Nusse, 2005; Zeng et al., 2008]. Such occurrences may be the result of the different models utilized combined with the common approach of overexpressing and/or utilizing exogenous purified recombinant protein for one or several Wnt/β-catenin pathway components. Therefore, results from these different models need to be carefully evaluated for the conclusions that can be drawn.

A second group [Piao et al., 2008; Yum et al., 2009] has recently published data using various truncated LRP6-ICD expression constructs. The truncated constructs were 40 residues or less and contained only the first PPP(S/T)P motif. Although the data obtained with this highly truncated construct are interesting, they are difficult to compare with the data obtained with the longer LRP6-ICDs which contain all 5 PPP(S/T)P motifs and the complete C-terminal of the protein as used in the study by Cselenyi et al. [2008] and in the current study.

In summary, the results of these studies indicate that LRP6-ICD is a biologically functional protein capable of cooperatively interacting in the Wnt/β-catenin pathway as a positive regulator, as well as acting as a general GSK3β effector protein. We show the non-membrane anchored LRP6-ICD, in contrast to the membrane anchored LRP6 intracellular domain, does not have to be phosphorylated by GSK3β at its PPP(S/T)P motif to stabilize cytosolic β-catenin and activate as well as amplify Wnt-mediated TCF/LEF-1 activation. Further, we show that LRP6-ICD interacts

with and attenuates GSK3β activity and this interaction/attenuation of GSK3β is not dependent upon GSK3β phosphorylation of the LRP6-ICD PPP(S/T)P. Overall it is clear that LRP6 is a dynamic, multi-functional protein whose function and regulation may vary depending upon its form/configuration and localization as well as interaction with other proteins.

ACKNOWLEDGMENTS

We thank Dr. P. Davies and Dr. L. Binder for providing tau antibodies and Dr. R. Grosschedl for the LEF-1 construct. We thank Philip Dolan for advice and discussion during the course of this project. We also thank Dr. William Tank and Carol Sterling for providing us facilities for the ^{32}P assays.

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