THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 22, pp. 15299–15307, May 29, 2009

Cooperative Folding and Ligand-binding Properties of LRP6 β-Propeller Domains*

Received for publication, September 19, 2008, and in revised form, March 26, 2009 Published, JBC Papers in Press, March 31, 2009, DOI 10.1074/jbc.M807285200

Chia-Chen Liu[1], **Chelsea Pearson, and Guojun Bu**[2]

From the Departments of Pediatrics and of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

Wnt/β-catenin signaling controls cell growth during development, and its misregulation in adults can cause human diseases. LRP6, the essential co-receptor for the Wnt pathway, consists of four β-propeller domains flanked by epidermal growth factor repeats in its extracellular region. To understand the maturation and ligand-binding properties of individual BP domains, we generated soluble receptor consisting of individual BPs, as well as combinations of these domains. We show that BP1, BP2, and BP4 each can be folded and secreted, and their secretion was enhanced by co-expression of Mesd, a molecular chaperone essential for LRP6 folding and maturation. BP3 is not secreted when expressed on its own or in combination with BP2 or BP1 and 2 (BP12); however, folding and secretion of BP3 is vastly enhanced when expressed together with BP4. Similar cooperative folding and maturation was observed between BP1 and BP2. These results suggest that BP1 forms a functional folding unit with BP2, whereas BP3 folds together with BP4. Using these BP constructs, we also found that BP12 and BP34 constitute independent ligand-binding domains capable of binding Wnt3a, Dkk1, and Mesd. The ability of Mesd to block the binding of both Wnt3a and Dkk1 to LRP6 enables this specialized chaperone to function as a Wnt signaling modulator. Together, our studies reveal unique properties of the LRP6 BP domains and provide novel tools to understand LRP6 function in ligand binding and Wnt signaling. Our results also support the development of soluble LRP6 receptors and Mesd as potential therapeutic molecules that target Wnt signaling.

The Wnt signaling pathway is involved in various differentiation events during embryonic development and can lead to tumor formation when aberrantly activated (1–3). Wnts are secreted glycoproteins that engage two cell surface receptors: seven transmembrane receptors of the Frizzled family and low density lipoprotein receptor (LDLR)[3]-related protein 5/6 (LRP5/6). In the absence of Wnt ligands, β-catenin is phosphorylated by the multiprotein degradation complex, Axin-APC-GSK3β, which marks it for ubiquitination and degradation by the proteasome. Wnt signaling thus stabilizes β-catenin, which in turns translocates to the nucleus and associates with TCF/LEF transcription factors. This interaction leads to the transcription of Wnt target genes, which are involved in the regulation of cell proliferation and development (4).

LRP6, a type I transmembrane protein of LDLR family, is an essential co-receptor for canonical Wnt signaling. Due to its indispensable role in Wnt signaling during early development, mice deficient in *LRP6* are perinatal lethal and exhibit composite defects that resemble those phenotype caused by the mutations in several Wnt genes (5). Recently, LRP6 mutations were also found to be directly associated with human diseases, including coronary artery disease, hyperlipidemia, and osteoporosis (6–7), suggesting the importance of proper LRP6 expression and regulation. The extracellular region of LRP6, which is critical in ligand-dependent regulation of Wnt signaling is composed of four distinct YWTD β-propeller/EGF domain pairs (BPs), followed by three LDLR type A repeats (3). Although the BP domains in the LDL receptor function to facilitate endosomal release of ligands, several studies have shown that the LRP6 BP domains actually bind ligands and comprise the functionally distinct ligand-binding domains (3). For example, although the predicted structures of different BP domains display significant homology (8), it has been shown that the second BP domain of LRP6 can functionally replace the BP domain of LDLR to induce ligand dissociation at low pH, whereas the fourth BP cannot (9), implicating the functional diversity among individual LRP6 BPs.

Dkk1, the founding member of the *dickkopf* (*Dkk*) family and a secreted antagonist for Wnt signaling, was identified as an embryonic head-inducer in *Xenopus* development (10). Distinct from several families of secreted Wnt antagonists that bind Wnts, including the secreted Frizzled-related protein family, Dkk1 does not bind Wnts but exhibits high affinity to LRP5/6 through which Dkk1 serves as a specific antagonist for Wnt/β-catenin pathway (11–13). It was proposed that Dkk1 inhibits Wnt signaling probably by preventing Frizzled-LRP5/6 complex formation in response to Wnt (13), a mechanism supported by mouse genetic studies (14). Alternatively, the inhibition may result from Dkk1 binding to LRP6 together with a transmembrane protein Kremen, which triggers rapid internalization and down-regulation of LRP6 from the cell surface (15), although a recent study has challenged this possibility (16). Given that Dkk1 plays a crucial role in regulating bone density,

* This work was supported, in whole or in part, by National Institutes of Health Grant CA100520. This work was also supported by a grant from the Midwest Regional Center for Excellence in Emerging infections Diseases (to G. B.).

[1] Partially supported by a Cancer Biology Pathway fellowship from the Washington University Siteman Cancer Center.

[2] To whom correspondence should be addressed: Washington University School of Medicine, Campus Box 8208, 660 South Euclid Ave., St. Louis, MO 63110. Tel.: 314-286-2860; Fax: 314-286-2894; E-mail: bu@wustl.edu.

[3] The abbreviations used are: LDLR, low density lipoprotein receptor; LRP5/6, LDLR-related proteins 5/6; BP, β-propeller; EGF, epidermal growth factor; Mesd, mesoderm development; CM, conditioned medium; PBS, phosphate-buffered saline; Co-IP, co-immunoprecipitation; CRD, cysteine-rich domain.

joint remodeling, and suppression of tumor formation, and is implicated in several human diseases such as osteoporosis and cancers (17), further understanding of the mechanism of Dkk1-LRP6 interaction may raise future therapeutic approaches for interfering with their interactions.

A specialized chaperone for members of LDLR family, termed Mesd (mesoderm development) in mouse and Boca in *Drosophila*, was discovered due to its requirement for the proper folding of LRP5/6 and their *Drosophila* homolog Arrow (18–19). In the absence of Mesd, LRP5/6 were found to be retained in the ER, failing to reach the cell surface. Consequently, the mice with *Mesd* deficiency were embryonic lethal and show phenotypes resembling that seen in *Lrp5/6*-deficient or *wnt3*$^{-/-}$ mutant mice (5). Additionally, it was demonstrated that Mesd/Boca is specifically required for the maturation of BP domains within LDLRs for their trafficking through the secretory pathway (19). Given that the β-propeller/EGF clusters account for the majority of the extracellular regions of LRP5/6, studying how Mesd interacts with LRP5/6 is especially intriguing. Our previous studies have shown that Mesd can also bind to mature LRP5/6 at the cell surface and antagonizes Dkk1 binding to these receptors (20). The structure of Mesd was predicted to consist of a central folded domain flanked by unstructured N- and C-terminal regions, which are required for maturation of LRP6 (21). It has been proposed that, as a specialized chaperone, these flexible structures of Mesd may confer advantage to the recognition of distinct BP pairs that may possess functional difference (21).

Wnt/β-catenin signaling is tightly modulated by secreted ligands/antagonists that bind to LRP6 at the cell surface. Previous studies using deletion approach mapped Wnt ligands binding to the first two BP domains from the N terminus (12), whereas Dkk1 was shown to interact with the third and fourth BP pairs of LRP6 (13). Currently, the combinations of LRP6 BP regions that are functional ligand binding units, and the requirement for Mesd in the folding of each BP have not been systematically examined. We are interested in dissecting the dependence of proper folding of individual or combinations of LRP6 BP domains on Mesd and understanding which BP domains are sufficient for binding to several known LRP6 ligands, including Wnt3a, Dkk1, and Mesd.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant Dkk1 protein and mouse recombinant Wnt3a protein were from R&D Systems. The preparation of recombinant Mesd protein was described previously (20). Human LRP6 antibodies (R&D), Wnt3a antibodies (R&D), Dkk1 antibodies (R&D), anti-FLAG antibodies (Sigma), anti-Myc antibodies (Sigma), or anti-actin antibodies (Sigma) were used according to manufacturers' instructions. Polyclonal rabbit anti-Mesd antibody was produced by immunizing rabbits with purified Mesd protein. Monoclonal anti-HA antibody has been described before (22).

Cell Culture and Transfection—BHK570 were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum and 1% L-glutamine, and maintained at 37 °C in humidified air containing 5% CO_2. For transient transfection, BHK570 cells were transfected with various plasmids at 90% confluence using Lipofectamine 2000 according to the manufacturer's instructions. For Mesd knockdown and BP secretion studies, BHK570 cells were grown to 60–70% confluence, and media were replaced with Dulbecco's minimal essential medium supplemented with 1% fetal bovine serum and 1% L-glutamine. Cells were transfected with control or Mesd siRNA and indicated plasmid cDNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. Negative control siRNA were purchased from Ambion. Mesd siRNAs were synthesized from Invitrogen, and the sequences of siRNA oligonucleotides were as follows: 5′-CCAGGCAAACCUGAGAGCAUCUUGA-3′ and 5′-AGAAAGGGAAGACUCUGAUGAUGUU-3′.

Generation of LRP6 BP Constructs—The schematic representation of BPs is illustrated in Fig. 1. Briefly, human LRP6 cDNA (kindly provided by Dr. Christof Niehrs, Deutsches Krebsforschungszentrum, Heidelberg, Germany) was used as the template for PCR. Each BP was constructed by subcloning PCR products into the BamH1/XbaI sites of mLRP4T100 backbone whose construction has been described previously (23). BPs were placed C-terminal to signal peptides and HA epitope; each BP includes YWTD β-propeller and EGF repeat (residues 20–324 for BP1; 325–628 for BP2; 629–929 for BP3; 930–1243 for BP4; 20–628 for BP12; 325–929 for BP23; 629–1243 for BP34; 20–929 for BP123; 325–1243 for BP234; 20–1243 for BP1234, see Ref. 24 for amino acid numbering). All constructs derived from PCR were confirmed by sequencing.

Preparation of BPs and Dkk1 Conditioned Medium—BHK570 cells were transiently transfected with HA-tagged BP12, BP34, or BP1234 together with Mesd. 6 h later, media were replaced with Dulbecco's minimal essential medium supplemented with 1% fetal bovine serum and 1% L-glutamine. Secretion of soluble BP12, BP34, and BP1234 was allowed to proceed for 48 h. The culture media were collected and centrifuged for removal of cell debris and were concentrated 30-fold with Centriprep YM-30 (Amicon). The control conditioned medium (CM) was produced by exactly the same procedure as the preparation of BP CM except pcDNA was transfected into cells instead of HA-tagged BP constructs. The amount of BP proteins secreted in the CM was quantified by immunoblot using pure Fc-LRP6 protein (R&D) as standard. For preparation of Dkk1 CM, HEK293 cells were transfected with an Myc-tagged Dkk1 construct. Conditioned media were collected 48 h after transfection and concentrated 10-fold. All preparations of CM were aliquoted and stored at −80 °C. Total protein amount was measured with Bio-Rad protein assay.

Western Blotting—BHK570 cells were transiently transfected with pcDNA, HA-tagged BPs together with pcDNA or with FLAG-tagged Mesd. 6 h later, culture media were replaced with Dulbecco's minimal essential medium supplemented with 1% fetal bovine serum, and the secretion of BPs proceeded for 48 h. Cells were lysed at 4 °C for 30 min, and culture media were concentrated with Centricon YM-10 (Millipore) at 4 °C. Equal quantities of protein were subjected to SDS-PAGE under reducing conditions. Following transfer to Immobilon-P transfer membrane, successive incubations with indicated primary and horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) were carried out according to manu-

facturer's specification. The immunoreactive proteins were then detected using the ECL system.

Solid-phase Binding Assay—Briefly, 96-well plates were coated with recombinant Wnt3a (1 μg/ml), rDkk1 (10 μg/ml), or rMesd (100 μg/ml) in coating buffer (1.7 mM NaH_2PO_4-H_2O, 98 mM Na_2HPO_4-H_2O, 0.1% sodium azide, pH 7.4) at 4 °C overnight, and nonspecific sites were blocked with 2% bovine serum albumin in phosphate-buffered saline buffer (PBS). The wells were then incubated with control CM or BP CM containing equal amounts of BP1234, BP12, or BP34 for 18 h at 4 °C. After five washes with PBS, the plates were incubated with anti-HA antibody (1 μg/ml) for 1.5 h at 37 °C. After washing, incubation with horseradish peroxidase-conjugated secondary antibody was carried out for 1.5 h at room temperature. Following the final washing step, the reaction was revealed with TMB substrate (3,3′,5,5′-tetramethylbenzidine, Sigma) and was measured by an enzyme-linked immunosorbent assay reader at 650 nm. Recombinant human LRP6-Fc (R&D), which is capable of binding rDkk1, rWnt3a, and rMesd, in a functional enzyme-linked immunosorbent assay (data not shown) was used to bind the indicated coated proteins as positive control. Bovine serum albumin coating (2%) and secondary antibody alone were used as a negative control.

Co-immunoprecipitation—Control and BP CM were incubated with rWnt3a (1 μg/ml), rDkk1 protein (1 μg/ml), or rMesd protein (10 μg/ml) for 16 h at 4 °C. Then, the immunocomplexes were incubated with anti-HA antibody for 2 h at 4 °C and were precipitated with immobilized protein A-agarose beads for 45 min. The beads were washed twice with PBSc (PBS, 0.5 mM Mg^{2+}, 1 mM Ca^{2+}, 0.5% Triton X-100), followed by an extensive wash with PBS two times, and boiled in SDS sample buffer containing β-mercaptoethanol. The supernatants were subjected to SDS-PAGE and Western blotting. Protein A-horseradish peroxidase (Sigma) and horseradish peroxidase-rec-Protein G (Zymed Laboratories Inc.) were used for WB detection instead of the traditional horseradish peroxidase-conjugated secondary antibody to avoid the high background signals resulted from the recognition of heavy chain and light chain of the immunoprecipitation antibody (26).

Luciferase Reporter Assay—HEK293T/STF cells stably transfected with the SuperTOPFlash luciferase reporter construct, which contains 8 tandem copies of the TCF binding site (27) were plated in black 24-well plates. Twenty-four hours later, the medium was replaced with colorless L cell CM, Wnt3a CM, or Wnt3a CM with addition of various reagents at 37 °C for 16 h. Wnt signaling activation was measured using live cell imaging technique in which cells were imaged for bioluminescence using a charge-coupled device camera (IVIS 50, Xenogen).

RESULTS

Proper Folding and Secretion of BPs Are Facilitated by Co-expression of Mesd—Previous studies showed that β-propeller/EGF module requires Boca, the *Drosophila* homolog of Mesd, for their folding and trafficking through the secretory pathway (19). To investigate the folding properties and the requirement of Mesd for individual BP domains of LRP6, we generated HA-tagged soluble receptor constructs consisting of individual BPs, as well as combinations of these domains shown as single, double, triple, and quadruple β-propellers. Each BP monomer or multimer (referred as BP1, BP2, BP3, BP4, BP12, BP23, BP34, BP123, BP234, and BP1234; see Fig. 1*A*) contains YWTD β-propeller and C-terminal EGF repeats. The Mesd dependence of LRP6 BP1234 folding and trafficking was first examined. As expected, co-expression of Mesd enhanced folding and secretion of LRP6 BP1234 (Fig. 1, *B* and *C*), consistent with the results that Mesd promotes LRP6 expression on the cell surface (25). To investigate the minimal module required for secretion of BPs and their responsiveness to Mesd co-expression, we performed a secretion assay in which cells were transfected with single BPs with or without co-expression of Mesd. We found that BP1, BP2, and BP4 each can be folded and secreted into the medium of cultured cells, and that secretion of these proteins was dramatically enhanced by co-expression of Mesd (Fig. 2, *A* and *B*). However, we did not detect any secretion of BP3 even with co-expression of Mesd, suggesting that BP3 cannot fold properly on its own and was likely retained in the ER.

LRP6 BP12 and BP34 Constitute Functional Folding Units—Given that BP3 itself cannot fold properly, we next tested whether any other β-propeller/EGF pairs can be folded together with BP3. Interestingly, neither BP23 nor BP123 can properly fold and traffic through the secretory pathway (Fig. 3, *A* and *B*). These results indicate that BP3 not only cannot fold properly when expressed on its own or in combination with BP2, but it also interferes in the folding of BP12. Surprisingly, the folding and secretion of BP3 is vastly enhanced when expressed together with BP4 even in the absence of Mesd co-expression. Similar cooperative folding and maturation was observed between BP1 and BP2, because BP12 was folded and secreted much better than individual BP1 or BP2. As expected, the folding and secretion of both BP12 and BP34 was facilitated by Mesd (Fig. 3, *A* and *B*). Similar results were observed when these cDNAs were transfected into several other cell lines (*e.g.* Chinese hamster ovary and L cells) (data not shown). Consistent with the idea that LRP6 BP12 and 34 are separate folding units, BP2 impaired the folding of BP34 when expressed together with BP3 and -4, resulting in the apparent misfolding of BP234 even when Mesd was co-expressed (Fig. 3, *A* and *B*). Taken together, these results imply that BP1 forms a folding unit with BP2, whereas BP3 folds together with BP4.

To further examine whether endogenous Mesd is required for the folding and secretion of LRP6 BPs, we knocked down Mesd using an RNA interference approach and examined the effect on the folding and secretion of LRP6 BPs. As expected, depletion of endogenous Mesd resulted in a dramatic reduction in the secretion of all LRP6 BPs (Fig. 4). The intracellular BPs in the cell lysates also showed a decrease in expression upon Mesd siRNA treatment, which is consistent with a previous study (21). These data indicate that Mesd plays an indispensable role in facilitating the proper folding and secretion of LRP6 BPs.

LRP6 BP12 and BP34 Are Functional Ligand Binding Units Displaying Different Preferences for Individual LRP6 Ligands—Previous deletion studies suggested that the first and second β-propeller/EGF domains of LRP6 may interact with Wnt, whereas the third and fourth domains are involved in binding to Dkk1 (12–13). To evaluate the contribution of LRP6 BP12 and BP34 domains to ligand binding, we performed solid phase

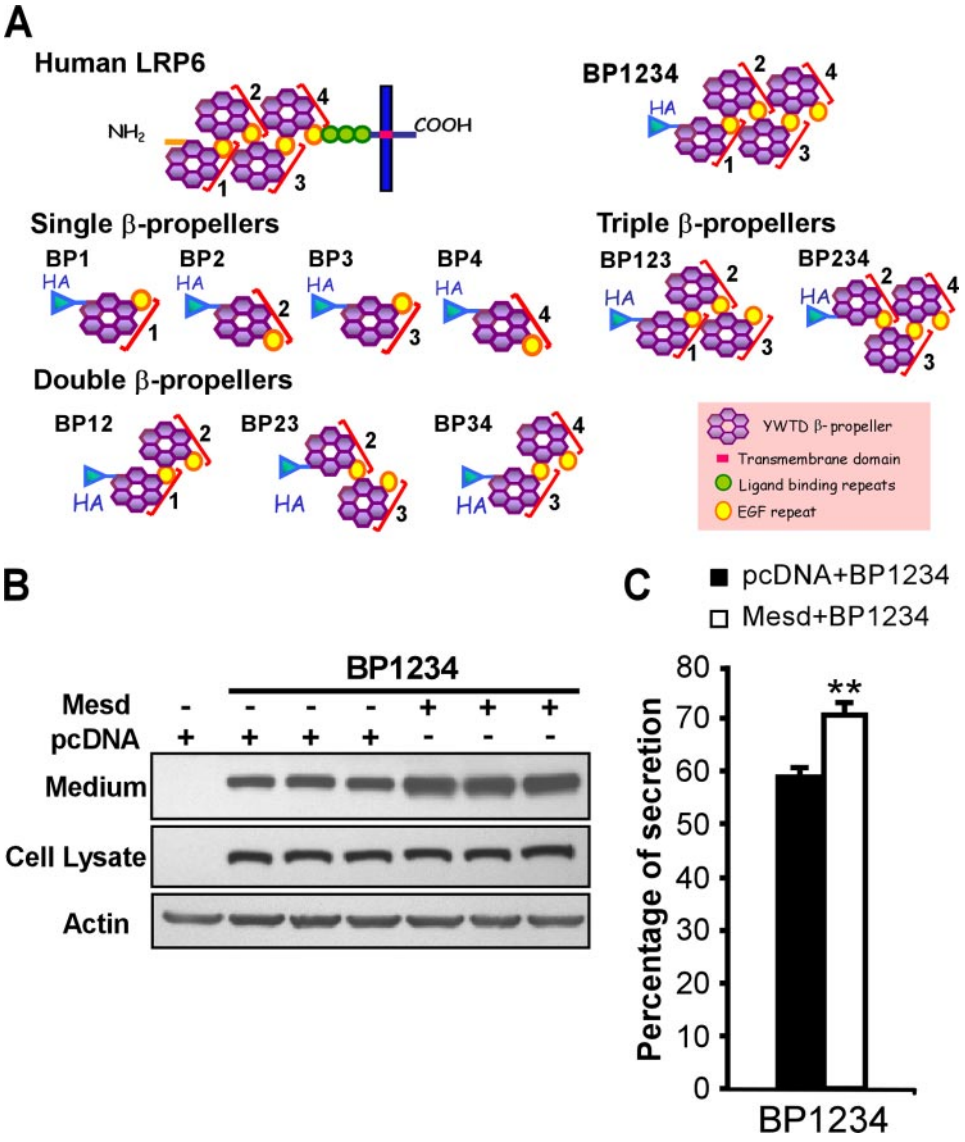

FIGURE 1. **Co-expression of Mesd enhances secretion of LRP6 β-propellers.** *A*, schematic representation of HA-tagged LRP6 BP constructs. *BP*, β-propeller. Each of the BPs (β-propeller/EGF module) is depicted in comparison to the full-length LRP6. The four BP domains are labeled with *1*, *2*, *3*, and *4*. *B*, soluble LRP6 containing all four BP domains (BP1234) was well secreted, and Mesd co-expression enhanced its maturation and secretion. BP1234 were transiently transfected into BHK570 cells, with or without Mesd co-transfection. Secretion of soluble BP1234 was allowed to proceed for 48 h, concentrated, followed by Western blot analysis of both conditioned media and cellular lysates. Data show one representative experiment done in triplicate, and β-actin was used as a loading control. *C*, the percentage of secretion was calculated by the quantitation of Western blots from three independent experiments. **, $p < 0.01$ compared with pcDNA co-transfected cells.

binding assay in which the plates were coated with rWnt3a, rDkk1, or rMesd followed by the analysis of binding ability of BP12 and BP34. Consistent with previous results on LRP6-binding properties (12–13, 20), BP1234, which consists of all four BP domains of LRP6, is capable of binding to Wnt3a ligand, its specialized chaperone Mesd, and the antagonist Dkk1 (Fig. 5, *A*–*C*). Surprisingly, both BP12 and BP34 are capable of binding all three ligands with slight preferences of BP12 for Wnt3a and Mesd, and BP34 for Dkk1 (Fig. 5, *A*–*C*). Control CM was used as a negative control to determine nonspecific binding. To further confirm the ligand-binding properties of BP12 and BP34, we assayed for their interaction with the indicated ligands in solution by co-immunoprecipitation (Co-IP). In agreement with the results of solid phase binding, Wnt3a (Fig. 6*A*), Dkk1 (Fig. 6*B*), and Mesd (Fig. 6*C*) proteins each co-immunoprecipitated with both BP12 and BP34. The amount of ligand that was co-immunoprecipitated with BPs was significantly above that observed with control CM. Taken together, these results demonstrate that BP1 and -2, and BP3 and -4 not only are separate folding units, but also represent distinct functional units in ligand binding.

LRP6 BP12 and BP34 CM Inhibit Wnt Signaling—Several soluble Wnt receptor domains, such as cysteine-rich domain (CRD) of fzd7, Frizzled8CRD fusion proteins, secreted Frizzled-related protein1, and dominant-negative form of LRP5 (28–29, 36), have been shown to act as secreted antagonists of Wnt signaling by sequestering Wnt ligands. To elucidate whether LRP6 BP12 and BP34 can also modulate Wnt signaling, we treated HEK293T/STF cells with Wnt3a CM together with control CM, BP12, or BP34 CM. As predicted on the basis of Wnt3a ligand-binding properties of BPs, treatment of BP12 and BP34 CM led to a ~60% reduction in luciferase activity likely resulted from sequestration of Wnt3a ligand (Fig. 7).

Mesd Antagonizes Ligand Binding to LRP6 β-Propeller/EGF Pairs—Given that Mesd and Wnt3a exhibit overlapping binding regions on LRP6, we examined whether Mesd is able to block Wnt3a binding to BPs. As expected, the presence of Mesd blocked Wnt3a binding to BP1234, BP12, and BP34 in the Co-IP experiment (Fig. 8, *A* and *B*). To confirm the above results, we examined the competition of Wnt3a and Mesd binding to BPs using a solid phase binding assay. Importantly, Mesd significantly competed with Wnt3a binding to BP1234, BP12, and BP34 (Fig. 8*C*). We next tested whether Mesd can block Dkk1 binding to LRP6 BPs using Dkk1 CM. Mesd can interfere with Myc-Dkk1 binding to BP1234, BP12, and BP34 (Fig. 8*D*), consistent with our previous finding that Mesd can antagonize Dkk1 binding to LRP6 on the cell surface (20).

Mesd Is a Novel Inhibitor for Wnt Signaling—Because Mesd binding regions were mapped to both BP12 and BP34 of LRP6, we next examined the effect of Mesd administration on Wnt signaling. As seen in Fig. 9, the presence of Wnt3a CM significantly increased Wnt signaling reporter activity when com-

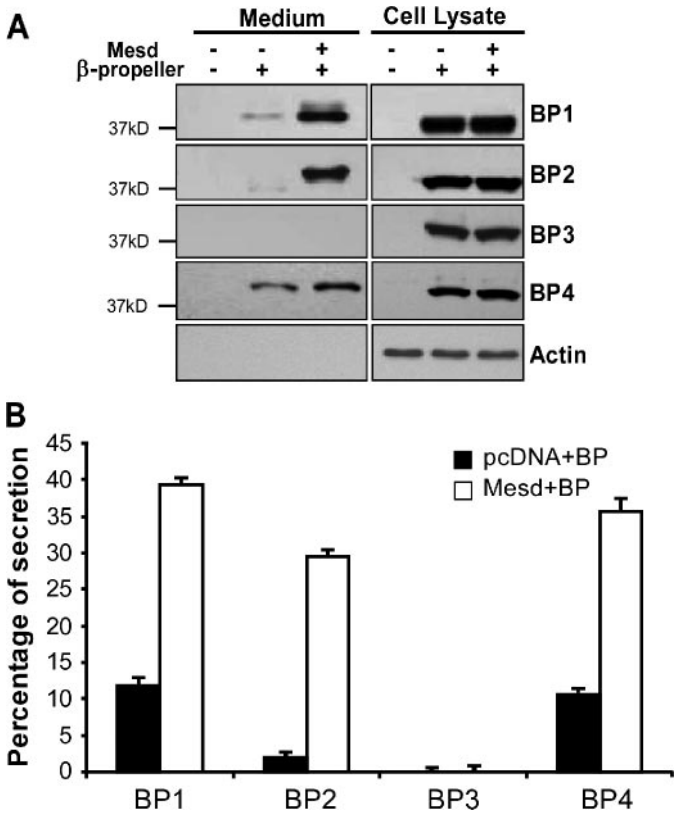

FIGURE 2. **Co-expression of Mesd facilitates single LRP6 β-propeller BP1, -2, and -4, but not BP3, folding and secretion.** *A*, BHK570 cells were transiently transfected with cDNAs for BP1, -2, -3, or -4, with co-transfection of either vector pcDNA or Mesd. *B*, quantification of secretion efficiency from Western blot analyses. Results shown in this figure and subsequent figures are representative of three independent experiments.

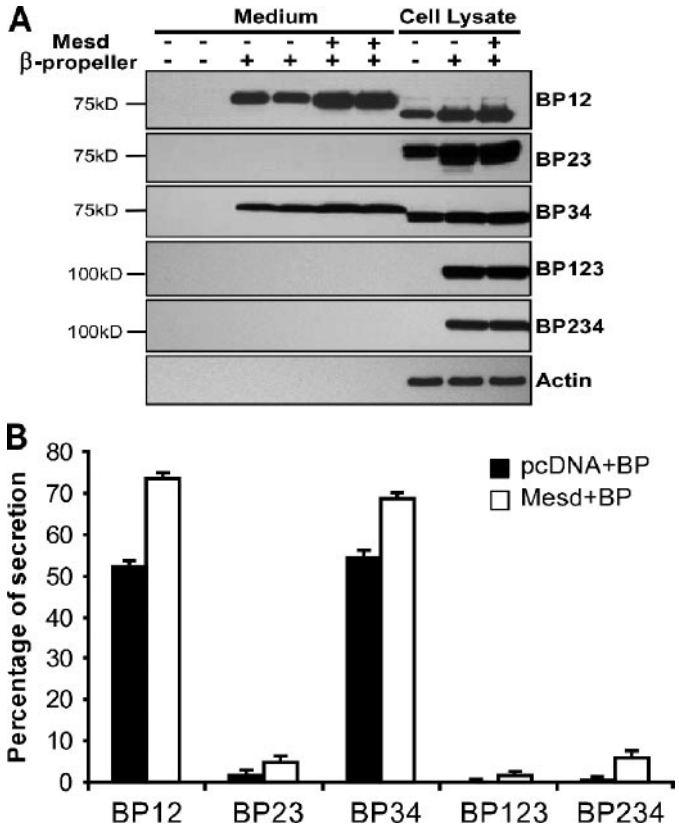

FIGURE 3. **LRP6 BP12 and BP34 are folding units whose secretion are enhanced by Mesd co-expression.** *A*, BP12, BP23, BP34, BP123, or BP234 were transiently transfected into BHK570 cells, with or without Mesd co-transfection. The band shown in vector-transfected cells in cell lysate represents a nonspecific band, which co-migrated with BP12, BP23, or BP34. *B*, quantification of secretion efficiency from Western blot analyses. The secretion experiments were performed as described above. Note that cooperative folding and maturation were observed between BP1 and -2 and BP3 and -4, and that their secretion is enhanced by Mesd, suggesting that BP1/2 and BP3/4 comprise functional folding units.

pared with the control L cell CM. Mesd protein inhibited Wnt3a-induced Wnt signaling by up to 50% (Fig. 9) likely by serving as a receptor antagonist to block Wnt ligand binding to LRP6 (Fig. 8, *A–C*). Both truncated recombinant Mesd protein (amino acids 150–195) and a synthetic Mesd peptide (amino acids 155–191), each containing the C-terminal region of Mesd that was previously shown to be necessary and sufficient for LRP6 binding (20), also inhibited Wnt3a-induced Wnt signaling. As a positive control, Dkk1, a potent inhibitor for LRP5/6-mediated Wnt signaling, inhibited Wnt signaling by >95%. Most interestingly, Mesd can also block Dkk1-induced Wnt signaling inhibition in which Wnt signaling was partially restored when both Dkk1 and Mesd peptide were applied (Fig. 9). Similar results were obtained when we assayed for free β-catenin using the glutathione *S*-transferase-E-cadherin binding assay (41) (data not shown). The observation, that Mesd is able to inhibit both Wnt3a- and Dkk1-induced effects on Wnt signaling, is consistent with the above results that Wnt3a, Dkk1, and Mesd probably share common or overlapping binding sites on LRP6 (Figs. 5 and 6). Together, these results demonstrate that Mesd is a novel antagonist for LRP5/6-mediated Wnt signaling.

DISCUSSION

Wnt/β-catenin signaling plays an important role in controlling cell proliferation and cell fate during embryogenesis and maintaining homeostasis in adult tissues; hence, the abnormal regulation of Wnt signaling results in a variety of diseases, including cancers and bone diseases (1–3, 30). The proper folding and trafficking of LRP5/6 to the cell surface is essential for the transduction of Wnt signals. In this study, we extensively analyzed the folding and ligand-binding properties of LRP6 BPs. We showed that BP1 forms functional folding and ligand-binding domain with BP2. Similarly, BP3 forms a functional domain with BP4. Efficient secretion of these BP pairs, even in the absence of Mesd co-expression, suggests that individual BPs may serve as "intra-domain chaperones" to promote the folding of BP pairs. The cooperative folding may also initiate structural interactions within their BP pairs which ultimately serve as functional ligand-binding domain within mature receptors.

LRP5/6 has four β-propeller/EGF modules and three LDL-A ligand binding repeats. The formation of correct disulfide bonds during receptor folding presents a challenging task for ER chaperones. In similarity to the function of RAP, another specialized molecular chaperone for the members of the LDLR family, Mesd may primarily inhibit indiscriminate disulfide bond formation, in particular among different domains within the receptors during and after their translation (23, 31). Domains translated early may complete their folding prior to

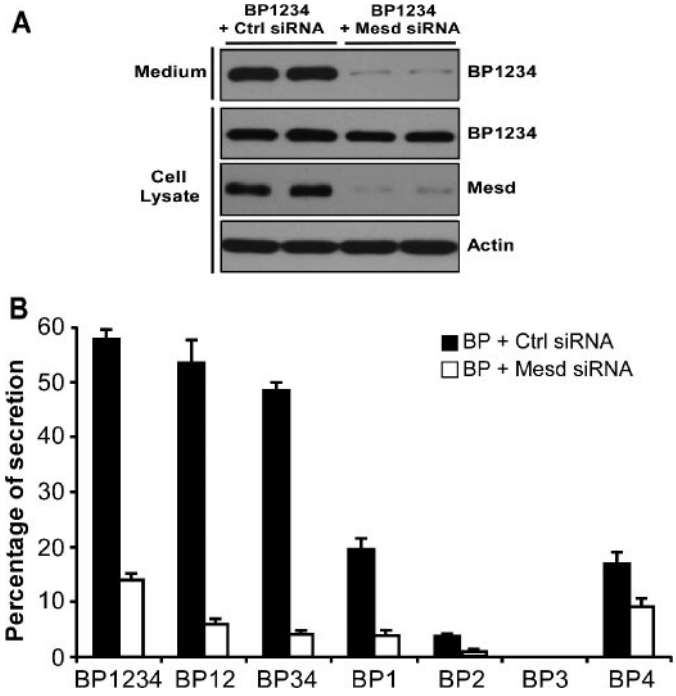

FIGURE 4. **Knockdown of endogenous Mesd impairs the folding and secretion of LRP6 BPs.** *A*, BHK570 cells were transfected with Mesd siRNA or control siRNA and cDNAs for BP1, -2, -3, and -4, BP12, BP34, or BP1234. Secretion of BPs was analyzed as described in the previous figures. A representative result of LRP6 BP1234 is shown. Secretion of this BP was strongly suppressed when the endogenous Mesd was knocked down. *B*, quantification of secretion efficiency of LRP6 β-propellers from Western blot analyses. Values are the average of triple determinations from at least two independent experiments with the S.D. indicated by *error bars*.

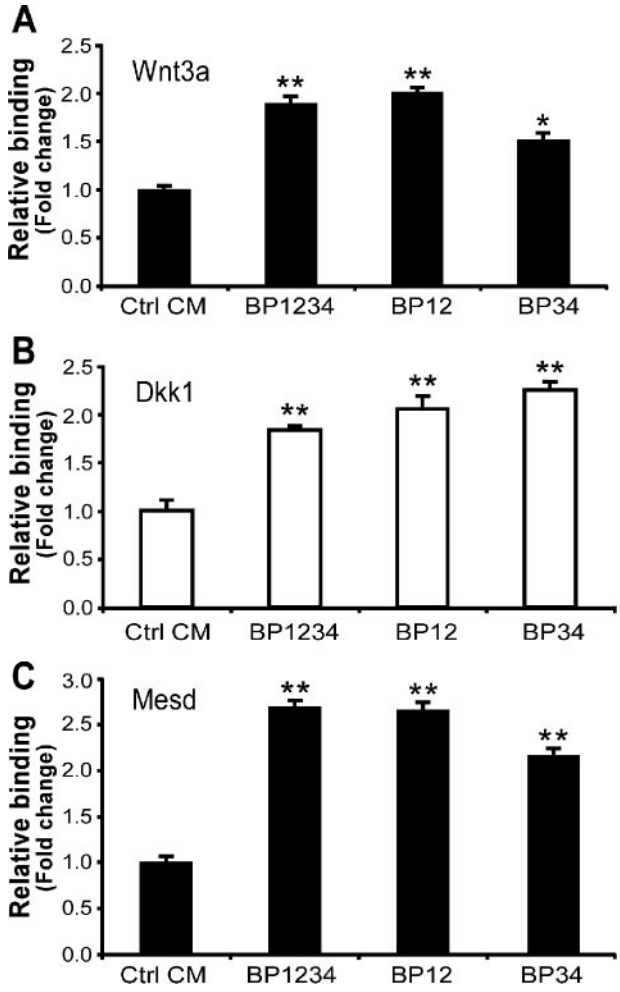

FIGURE 5. **LRP6 BP12 and BP34 are functional units displaying different preferences for the LRP6 ligands: Wnt3a, Dkk1, and Mesd.** Solid phase binding assays in which *A*, recombinant Wnt3a, *B*, recombinant Dkk1, and *C*, recombinant Mesd were coated onto the 96-well plates, followed by the incubation of control, LRP6 BP1234, BP12, or BP34 CM. *CM*, conditioned media. The amount of secreted BP1234, BP12, and BP34 in CM were previously quantified by in comparison to Fc-LRP6 pure protein (R&D) with known concentrations in Western blot analysis, and the same amount of BPs was applied. Values are the average of triple determinations with the S.D. indicated by *error bars*. Note that Wnt3a and Mesd display a preference for BP12, whereas Dkk1 binds better to BP34. *, $p < 0.05$; **, $p < 0.01$ compared with control CM.

the translation of subsequent domains. In case of LRP6, BP1 and -2 may be bound and folded by Mesd chaperone during or immediately after their translation, followed by the folding of BP3 and -4, which maintains the proper conformation and prevents the incorrect intermolecular cross-linking of the newly synthesized receptors.

Given that soluble and membrane-bound proteins generally undergo similar post-translational folding and modification along the secretory pathway, soluble receptor systems described in this study may provide a useful strategy to analyze the folding process of membrane-bound protein such as LRP6. Similar types of analyses using soluble recombinant proteins in the folding and trafficking of other membrane proteins have been performed (23, 39). In the case of soluble LRP6, reduced secretion of BPs likely results from the misfolded proteins, which aggregate and are retained in the ER. In addition, this system can also be applied to dissect the structural elements that are essential for the chaperone function of Mesd in receptor folding. For example, previous studies have determined the core region structure of Mesd by NMR (32), and a predicted unstructured region of Mesd has been suggested to be required for Mesd function (21). It will be of interest to examine whether a given domain or certain amino acids within Mesd are crucial for its chaperone function on individual BPs or their combinations. It is likely that the chaperone function requires multiple interactions between Mesd and the receptors to maintain a proper conformation for correct receptor folding. Furthermore, a recent study showed that a spontaneous point mutation located in the third BP of LRP6 results in reduced affinity of LRP6 mutant protein to Mesd. Due to this decreased association, the targeting of LRP6 to the cell surface and Wnt signaling were impaired, which leads to low bone mass phenotype in mice (7). These results further highlight the importance of understanding the interaction between LRP6 and Mesd.

In our current study, we showed that Mesd enhances the folding and secretion of BP1, BP2, BP4, BP12, BP34, and BP1234. However, in a previous study (19), it was shown that BP2 from Arrow (*Drosophila* homolog of LRP5/6) is not secreted in wild-type cells, which express Boca (*Drosophila* homolog of Mesd). There are several possibilities that might account for the difference between ours and their studies. First, the folding and secretion mechanism of human LRP6 BP may be different from that of *Drosophila* Arrow. Second, mammalian cells were used as the experimental system in the current study for LRP6, whereas insect cells were used in previous study

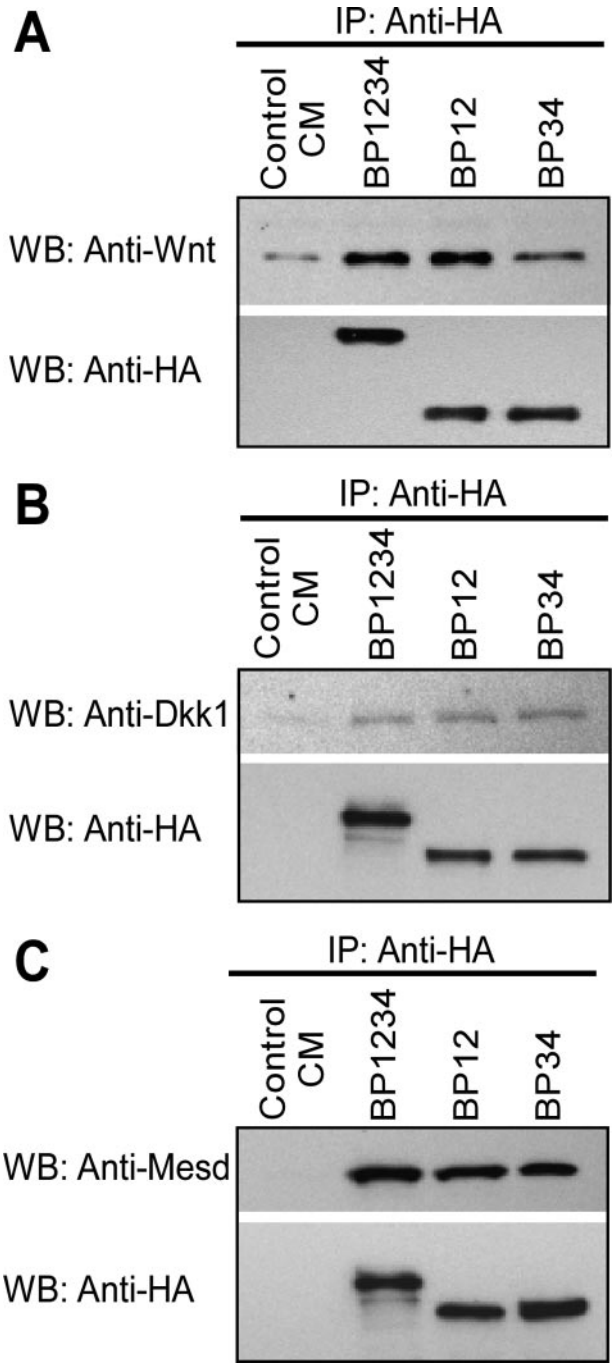

FIGURE 6. **LRP6 BP1234, BP12, and BP34 directly interact with LRP6 ligands Wnt3a, Dkk1, and Mesd.** BP CM was incubated with recombinant Wnt3a (*A*), recombinant Dkk1 (*B*), and recombinant Mesd (*C*), immunoprecipitated with anti-HA antibody or normal IgG (data not shown), and then probed with anti-Wnt3a, anti-Dkk1, or anti-Mesd antibodies to detect the indicated proteins. WB was analyzed via 7.5% (*A*) or 15% (*B* and *C*) SDS gels under reducing conditions. *IP*, immunoprecipitation; *WB*, Western blot.

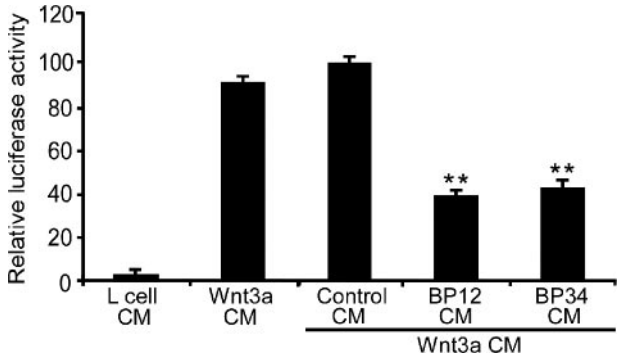

FIGURE 7. **LRP6 BP12 and BP34 CM inhibit Wnt signaling.** HEK293T/STF cells were incubated with L cells CM, Wnt3a CM, or Wnt3a CM along with control or LRP6 BP CM at 37 °C for 16 h. BP12 and BP34 CM contain ~30 μg of BP12 or BP34 protein, respectively, quantified by Western blot. Control CM containing equal amounts of total protein as BP CM was added to cells as negative control. Values are the average of triple determinations with the S.D. indicated by *error bars*. Note that both LRP6 BP12 and BP34 CM partially inhibit Wnt3a-induced Wnt signaling. **, $p < 0.01$ compared with control medium.

for Arrow. Third, our present study includes both overexpression and knockdown approaches, whereas only knockdown approach was employed in the previous study. Future studies are needed to further define the folding and secretion properties of LRP6 BPs. Nonetheless, both our study and the previous report (19) have clearly demonstrated the unique properties of individual LRP6 BPs and their dependence on Mesd/Boca to fold their complex structures.

LRP5/6 receptors are also subject to modulation by the interactions of their ligands/antagonists, through which Wnt signaling is regulated. Previous deletion studies showed that LRP6 BP12 and BP34 each represent separate binding sites for either Wnt or Dkk1, respectively (12, 13). Given that Dkk1 is a high affinity ligand for LRP6 (K_d = 0.3 nM) and its inhibition of Wnt signaling strictly depends on its binding to LRP5/6 (13), it is likely that Dkk1 and Wnt compete for the same binding site on LRP6. However, we cannot rule out the possibility that Dkk1 binding to LRP6 leads to conformational changes that prevent LRP6 interaction with Wnt. In addition, several LRP5 mutations associated with high bone density syndromes and previously shown to prevent Dkk from binding to LRP5 are located in the first BP domain (33) rather than domain 3 or 4. This suggests that BP34 may not be the only site for Dkk1 binding on LRP5, and this possibility was confirmed by the observation that Dkk1 can also bind to the BP12 of LRP5 (34), and probably to LRP6 as well, because LRP6 is also involved in bone metabolism (7). In the present study, we demonstrated that LRP6 BP12 and BP34 are functional units displaying different ligand binding preferences. Using direct binding assays, we showed that, despite BP12 and BP34 favoring binding to Wnt and Dkk1, respectively, the other BP domains also exhibit affinity to these two ligands. Our observation is in agreement with a recent finding that Dkk1 antagonizes Wnt signaling by direct competition with Wnt proteins for LRP6 binding (13, 16). The precise binding affinity and mechanism of BP12 or BP34 to either Wnt3a or Dkk1 require further investigation.

Given that dysregulated Wnt signaling has been implicated in several human diseases, developing modulators as therapeutic agents to antagonize or up-regulate Wnt signaling is of growing interest. For example, extracellular Wnt antagonists, including secreted Frizzled-related proteins (34), anti-Wnt antibodies (35), and soluble Wnt receptor F8CRDhFc (36) aiming to down-regulate the Wnt pathway, have been shown to reduce cancer cell growth in several Wnt ligand-driven tumor models. Conversely, suppression of Dkk1 levels or binding capacity to up-regulate Wnt signaling was used as a therapeutic strategy to increase bone mass in osteoporosis, and cancer-in-

duced bone loss (40). Similarly, soluble BP12 and BP34, which are capable of sequestering Wnt and Dkk1, may be applied to modulate Wnt signaling in a context-dependent manner. Here, we show that BP12 and BP34 CM are able to sequester Wnt3a and attenuate Wnt3a-induced signaling activation (Fig. 7). Similarly, BP12 and BP34 may sequester Dkk1 to suppress Dkk-mediated antagonism of Wnt signaling. Although BP CM was used in this study due to the difficulty of obtaining sufficient amount of purified BP proteins produced by mammalian cells, our finding suggests the potential of utilizing soluble BPs as Wnt signaling modulators.

Numerous studies have demonstrated that the Wnt signaling pathway also plays a crucial role in regulating the self-renewal and differentiation of stem cells (37). Complete blockage of Wnt pathway by systemic administration of Dkk1 has been shown to result in severe side effects on the intestines of mice due to its interference with intestinal stem cell renewal (38). Thus, identifying effective Wnt signaling inhibitor therapeutics without disrupting stem cell functions is still a challenge for present-day drug development. Previously, we found that Mesd is capable of binding mature LRP6 at the cell surface and antagonizing ligand binding (20). In this study, we demonstrated that Mesd can interact with both BP12 and BP34 of LRP6, which is in full agreement with the notion that Mesd is required for the proper folding of these β-propeller/EGF pairs. Most significantly, Mesd can simultaneously suppress Wnt signaling and restore Dkk1-mediated inhibition. These opposing effects result in tuning of the Wnt signaling to an intermediate level (Fig. 9). We

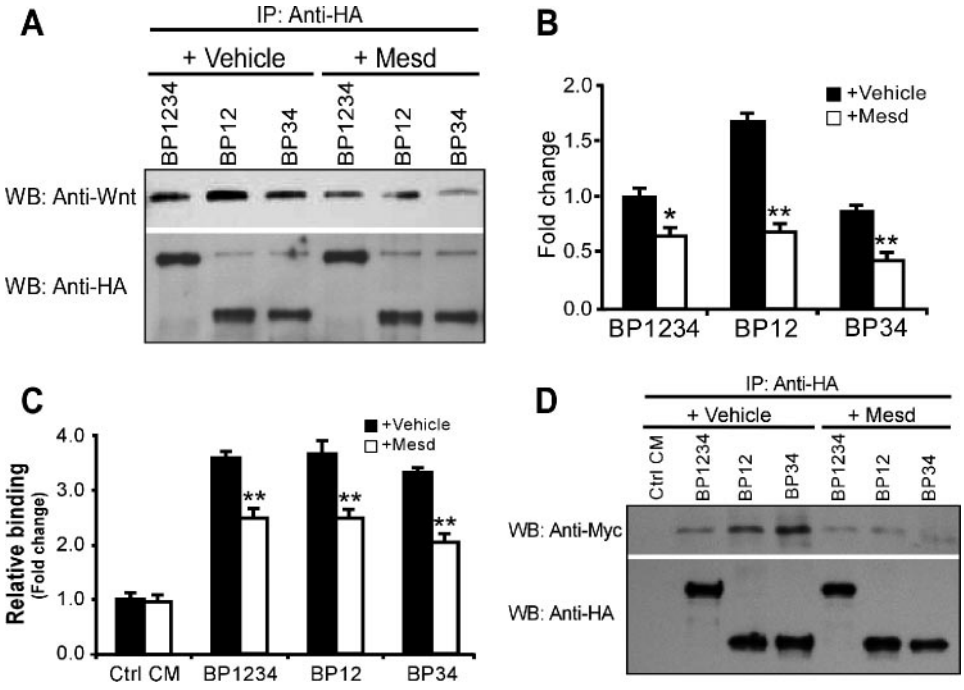

FIGURE 8. **Mesd antagonizes Wnt3a binding to LRP6 BP1234, BP12, and BP34.** Recombinant Mesd (1 μM) is able to compete for rWnt3a (1 μg/ml) binding to BPs in co-immunoprecipitation (Co-IP) (*A*) and solid phase binding assays (*C*). *B*, quantification of results from Co-IP. *D*, recombinant Mesd (1 μM) is able to compete for Myc-Dkk1 binding to BPs in Co-IP. *IP*, immunoprecipitation; *WB*, Western blot.

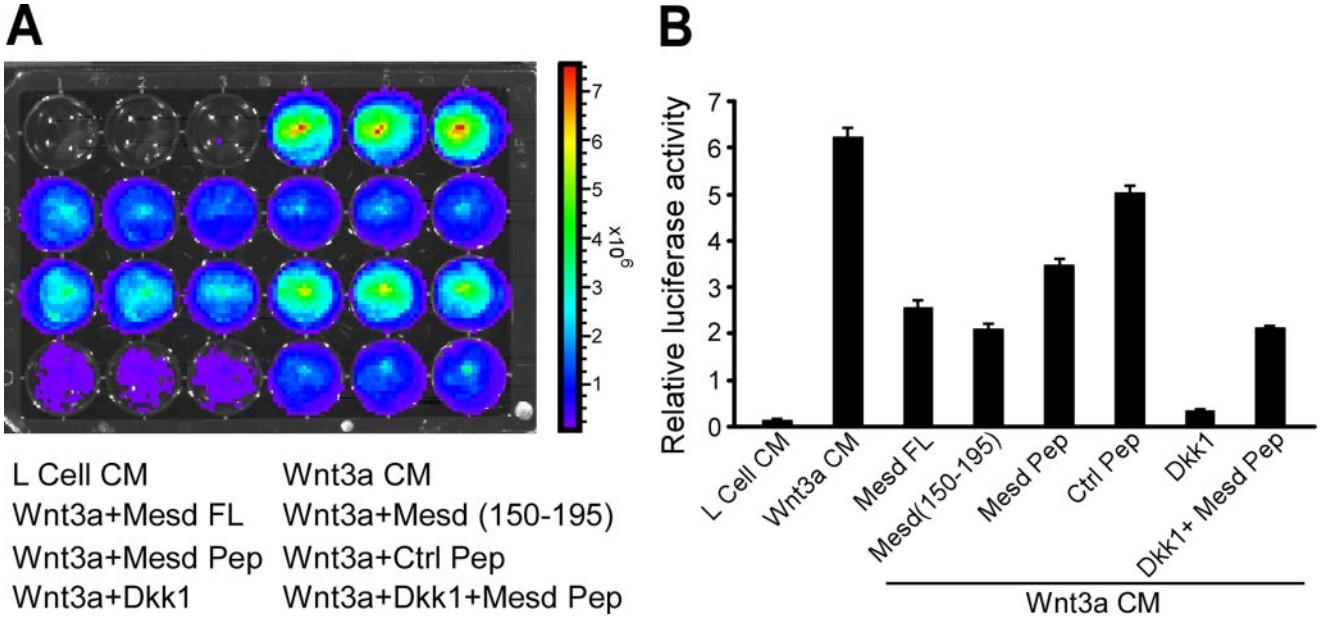

FIGURE 9. **Mesd is a novel inhibitor for Wnt signaling.** *A*, HEK293T cells stably expressing SuperTopFlash reporter were incubated with L cell CM, Wnt3a CM, or Wnt3a CM together with Mesd protein (1 μM), Mesd (150–195) protein (1 μM), Mesd peptides (1 μM), or Dkk1 (10 nM) as indicated in triplicates for 16 h at 37 °C. Cells were then imaged for bioluminescence using a IVIS charge-coupled device camera. *B*, quantitation of the triplicate samples in *A*. The data shown are representative of three independent experiments. Note that Mesd protein and peptide inhibited Wnt signaling and partially restored Wnt signaling in the presence of both Wnt3a and Dkk1.

found that higher concentrations of Mesd do not further suppress Wnt signaling (data not shown). This observation makes Mesd an attractive therapeutic candidate, because it may effectively inhibit a significant amount of Wnt signaling while maintaining a minimal level sufficient to support stem cell function. Together, these results demonstrated that Mesd is a novel antagonist for LRP5/6-mediated Wnt signaling.

Acknowledgment—We thank Dr. Todd Zankel at Raptor Pharmaceutical for critical reading of this manuscript and for providing valuable reagents.

REFERENCES

1. Wodarz, A., and Nusse, R. (1998) *Annu. Rev. Cell Dev. Biol.* **14,** 59–88
2. Lustig, B., and Behrens, J. (2003) *J. Cancer Res. Clin. Oncol.* **129,** 199–221
3. He, X., Semenov, M., Tamai, K., and Zeng, X. (2004) *Development* **131,** 1663–1677
4. Clevers, H. (2006) *Cell* **127,** 469–480
5. Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J., and Skarnes, W. C. (2000) *Nature* **407,** 535–538
6. Mani, A., Radhakrishnan, J., Wang, H., Mani, M. A., Nelson-Williams, C., Carew, K. S., Mane, S., Najmabadi, H., Wu, D., and Lifton, R. P. (2007) *Science* **315,** 1278–1282
7. Kubota, T., Michigami, T., Sakaguchi, N., Kokubu, C., Suzuki, A., Namba, N., Sakai, N., Nakajima, S., Imai, K., and Ozono, K. (2008) *J. Bone Miner. Res.* **23,** 1661–1671
8. Jeon, H., Meng, W., Takagi, J., Eck, M. J., Springer, T. A., and Blacklow, S. C. (2001) *Nat. Struct. Biol.* **8,** 499–504
9. Beglova, N., Jeon, H., Fisher, C., and Blacklow, S. C. (2004) *Mol. Cell* **6,** 281–292
10. Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C., and Niehrs, C. (1998) *Nature* **391,** 357–362
11. Bafico, A., Liu, G., Yaniv, A., Gazit, A., and Aaronson, S. A. (2001) *Nat. Cell Biol.* **3,** 683–686
12. Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A., and Niehrs, C. (2001) *Nature* **411,** 321–325
13. Semenov, M. V., Tamai, K., Brott, B. K., Kuhl, M., Sokol, S., and He, X. (2001) *Curr. Biol.* **11,** 951–961
14. MacDonald, B. T., Adamska, M., and Meisler, M. H. (2004) *Development* **131,** 2543–2552
15. Mao, B., Wu, W., Davidson, G., Marhold, J., Li, M., Mechler, B. M., Delius, H., Hoppe, D., Stannek, P., Walter, C., Glinka, A., and Niehrs, C. (2002) *Nature* **417,** 664–667
16. Semenov, M. V., Zhang, X., and He, Xi. (2008) *J. Biol. Chem.* **283,** 21427–21432
17. Niehrs, C. (2006) *Oncogene* **25,** 7469–7481
18. Hsieh, J. C., Lee, L., Zhang, L., Wefer, S., Brown, K., DeRossi, C., Wines, M. E., Rosenquist, T., and Joldener, B. C. (2003) *Cell* **112,** 355–367
19. Culi, J., Springer, T. A., and Mann, R. S. (2004) *EMBO J.* **23,** 1372–1380
20. Li, Y., Chen, J., Lu, W., McCormick, L. M., Wang, J., and Bu, G. (2005) *J. Cell Sci.* **118,** 5305–5314
21. Koduri, V., and Blacklow, S. C. (2007) *Biochemistry* **46,** 6570–6577
22. Li, Y., Marzolo, M. P., van Kerkhof, P., Strous, G. J., and Bu, G. (2000) *J. Biol. Chem.* **275,** 17187–17194
23. Bu, G., and Rennke, S. (1996) *J. Biol. Chem.* **271,** 22218–22224
24. Brown, S. D., Twells, R. C., Hey, P. J., Cox, R. D., Levy, E. R., Caskey, C. T., Todd, J. A., and Hess, J. F. (1998) *Biochem. Biophys. Res. Commun.* **248,** 879–888
25. Li, Y., Lu, W., He, X., and Bu, G. (2006) *FEBS Lett.* **580,** 5423–5428
26. Lal, A., Haynes, S. R., and Gorospe, M. (2005) *Mol. Cel. Probes* **19,** 385–388
27. Veeman, M. T., Slusarski, D. C., Kaykas, A., Louie, S. H., and Moon, R. T. (2003) *Curr. Biol.* **13,** 680–685
28. Zi, X., Guo, Y., Simoneau, A. R., Hope, H., Xie, J., Holcombe, R. F., and Hoang, B. H. (2005) *Cancer Res.* **65,** 9762–9770
29. Bafico, A., Liu, G., Goldin, L., Harris, V., and Aaronson, S. A. (2004) *Cancer Cell* **6,** 497–506
30. Li, Y., and Bu, G. (2005) *Future Oncol.* **1,** 673–681
31. Bu, G. (2001) *Int. Rev. Cytol.* **209,** 79–116
32. Kohler, C., Andersen, O. M., Diehl, A., Krause, G., Schmieder, P., and Oschkinat, H. (2006) *J. Struct. Funct. Genomics* **7,** 131–138
33. Boyden, L. M., Mao, J., Belsky, J., Mitzner, L., Farhi, A., Mitnick, M. A., Wu, D., Insogna, K., and Lifton, R. P. (2002) *New Engl. J. Med.* **346,** 1513–1521
34. Zhang, Y., Wang, Y., Li, X., Zhang, J., Mao, J., Li, Z., Zheng, J., Li, L., Harris, S., and Wu, D. (2004) *Mol. Cell. Biol.* **24,** 4677–4684
35. You, L., He, B., Xu, X., Uematsu, K., Mazieres, J., Fujii, N., Mikami, I., Reguart, N., McIntosh, J. K., Kashani-Sabet, M., McCormick, F., and Jablons, D. M. (2004) *Cancer Res.* **64,** 5385–5389
36. DeAlmeida, V. I., Miao, L., Ernst, J. A., Koeppen, H., Polakis, P., and Rubinfeld, B. (2007) *Cancer Res.* **67,** 5371–5379
37. Reya, T., and Clevers, H. (2005) *Nature* **434,** 843–850
38. Kuhnert, F., Davis, C. R., Wang, H. T., Chu, P., Lee, M., Yuan, J., Nusse, R., and Kuo, C. J. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 266–271
39. Singh, I., Doms, R. W., Wagner, K. R., and Helenius, A. (1990) *EMBO J.* **9,** 631–639
40. Tian, E., Zhan, F., Walker, R., Rasmussen, E., Ma, Y., Barlogie, B., and Shaughnessy, J. R. (2003) *N. Engl. J. Med.* **349,** 2483–2494
41. Bafico, A., Gazit, A., Wu-Morgan, S. S., Yaniv, A., and Aaronson, S. A. (1998) *Oncogene* **16,** 2819–2825