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Secreted Frizzled-related protein potentiation versus inhibition of Wnt3a/β-catenin signaling

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Abstract

Wnt signaling regulates a variety of cellular processes during embryonic development and in the adult. Many of these activities are mediated by the Frizzled family of seven-pass transmembrane receptors, which bind Wnts via a conserved cysteine-rich domain (CRD). Secreted Frizzled-related proteins (sFRPs) contain an amino-terminal, Frizzled-like CRD and a carboxyl-terminal, heparin-binding netrin-like domain. Previous studies identified sFRPs as soluble Wnt antagonists that bind directly to Wnts and prevent their interaction with Frizzleds. However, subsequent observations suggested that sFRPs and Frizzleds form homodimers and heterodimers via their respective CRDs, and that sFRPs can stimulate signal transduction. Here, we present evidence that sFRP1 either inhibits or enhances signaling in the Wnt3a/β-catenin pathway, depending on its concentration and the cellular context. Nanomolar concentrations of sFRP1 increased Wnt3a signaling, while higher concentrations blocked it in HEK293 cells expressing a SuperTopFlash reporter. sFRP1 primarily augmented Wnt3a/β-catenin signaling in C57MG cells, but it behaved as an antagonist in L929 fibroblasts. sFRP1 enhanced reporter activity in L cells that were engineered to stably express Frizzled 5, though not Frizzled 2. This implied that the Frizzled expression pattern could determine the response to sFRP1. Similar results were obtained with sFRP2 in HEK293, C57MG and L cell reporter assays. CRD_{sFRP1} mimicked the potentiating effect of sFRP1 inmultiple settings, contradicting initial expectations that this domain would inhibit Wnt signaling. Moreover, CRD_{sFRP1} showed little avidity for Wnt3a compared to sFRP1, implying that the mechanism for potentiation by CRD_{sFRP1} probably does not require an interaction with Wnt protein. Together, these findings demonstrate that sFRPs can either promote or suppress Wnt/β-catenin signaling, depending on cellular context, concentration and most likely the expression pattern of Fzd receptors.

Keywords

β-Catenin; Cysteine-rich domain; Frizzled; sFRP; Wnt

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1. Introduction

Wnts are secreted, lipid-modified glycoproteins that have many critical roles during embryonic development and promote tissue homeostasis in the adult. They regulate a wide range of cellular behavior including differentiation, proliferation, migration, survival, polarity and stem cell self-renewal [1–3]. Aberrant Wnt signaling is associated with several disorders, especially cancer [4]. The most extensively studied Wnt signaling mechanism is the canonical/β-catenin pathway, which is activated by Wnt binding to a member of the Frizzled (Fzd) receptor family and co-receptor LRP5 or LRP6 [5,6]. This results in the recruitment of Dishevelled to Fzd and Axin to phosphorylated LRP5/6, causing the dissociation of a β-catenin degradation complex in which Axin serves as a scaffold protein for β-catenin, GSK3, CK1α and APC. In the absence of Wnt, this complex mediates the sequential phosphorylation of β-catenin by CK1αand GSK3, leading to its ubiquitination and proteasomal degradation. Wnt stimulation enables the accumulation of hypophosphorylated β-catenin in the cytosol and its translocation into the nucleus, where it binds to TCF/LEF transcription factors and promotes the expression of Wnt/β-catenin target genes [5,6]. Constitutive activation of this pathway is common in many types of cancer [1,4]. Non-canonical Wnt signaling pathways associated with planar cell polarity or increases in intracellular calcium concentration are transduced by Fzds and/or other Wnt receptors or co-receptors, Ryk/Derailed and Ror1/2, without the involvement of LRP5/6 [6,7]. Various non-canonical Wnt signaling mechanisms have been reported to inhibit the β-catenin pathway by increasing β-catenin turnover or decreasing β-catenin/TCF association with DNA [8–10].

The secreted Frizzled-related proteins (sFRPs) comprise a family of five proteins in mammals that were first identified as antagonists of the Wnt/β-catenin pathway during embryonic development [11–13]. They contain a Fzd-type cysteine-rich domain (CRD) and a netrin-like motif [13]. The CRD of Fzds (CRD_{Fzd}) is primarily responsible for binding to Wnts [14,15], and initial deletion analysis suggested that the CRD of sFRPs (CRD_{sFRP}) also had Wnt-binding properties that accounted for its inhibitory activity [16]. However, subsequent experiments indicated that the avidity of Wnts for CRD_{sFRPs} is weaker than it is for CRD_{Fzds} [17] and Wnt binding to sFRPs was more closely associated with the netrin domain [18,19]. Furthermore, CRD_{sFRPs} and CRD_{Fzds} form homodimers and heterodimers, suggesting that CRD_{sFRP}/CRD_{Fzd} direct interaction could provide another mechanism for the regulation of signal transduction [20–22].

The sFRPs have a remarkable range of biological activities. Their antagonistic effect onWnt/β-catenin signaling suggests that they function as tumor suppressors [23,24]. This idea is reinforced by the epigenetic silencing of *SFRP* gene expression in a wide variety of cancers [25–27], and evidence that restoration of expression attenuated the tumor phenotype [23,28–30]. Alternatively, elevation of *SFRP* expression has been observed in some of the same malignancies, including breast [31], prostate [32,33] and renal cancer [34]. Consistent with this dichotomy, sFRP1 had a biphasic effect on β-catenin stabilization elicited by Wingless (the *Drosophila* ortholog of mammalian Wnt1), increasing β-catenin protein levels at low sFRP1 concentrations but inhibiting it at high concentrations [18]. In different cellular contexts, sFRP1 and sFRP2 have been shown to either increase or decrease β-catenin stabilization [35–38]. Furthermore, one study suggested that sFRP1 could stimulate the Wnt/calcium pathway via Fzd2 independent of endogenous Wnts [39]. In addition to these Wnt/Fzd-related activities, sFRPs also affect cell behavior by directly binding to proteases and regulating their activity [40–43], or binding to thrombospondin-1 to modulate cell adhesion and motility [44].

The present study was undertaken to better understand the factors that account for the ability of sFRPs to either potentiate or inhibit Wnt/β-catenin signaling. For this purpose, we tested the activity of purified recombinant proteins, primarily Wnt3a and sFRP1, on multiple cell lines and monitored various readouts of pathway activation including β-catenin protein stabilization, accumulation in the nucleus and transcriptional activity as measured by a promoter reporter assay and endogenous gene expression. Cell context was a major factor in determining the nature of the response to sFRP1. We tested the hypothesis that the expression of particular Fzds was pivotal in defining sFRP activity, and found that the ectopic expression of Fzd5 enabled sFRP1 and sFRP2 to potentiate Wnt3a/β-catenin signaling in a cell that otherwise only supported an inhibitory effect. Moreover, the CRD_{sFRP1} exhibited the potentiating activity, but little of the inhibitory activity displayed by full-length sFRP1.

2. Materials and methods

2.1. Cell culture

HEK293 cells (ATCC no. CRL-1573,Manassas,VA) and the HEK293/STF clonal line, kindly provided by Dr. Jeremy Nathans (Johns Hopkins University), were maintained in DMEM (cat. no. 11995, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (cat. no. 10438, Gibco, Grand Island, NY). HEK293/STF cells stably express a SuperTopFlash reporter with 7 tandem repeats of TCF binding sites [45]. C57MG cells, a gift from the Varmus lab, NCI, were grown in DMEM supplemented with 10% FBS and 10 μg/ml insulin (cat. no. 12585-014, Invitrogen). L929 fibroblasts (L cells) were maintained in MEM (cat. no. 11090, Gibco) supplemented with 10% FBS, 1% sodium pyruvate (cat. no. 11360-070, Gibco) and 1% MEM non-essential amino acids (cat. no.11140-050, Gibco). All cell lines were incubated at 37 °C with 5% CO_2.

2.2. Plasmids

Constructs encoding the human Fzd5 signal peptide, followed by two tandem repeats of the HA epitope and the downstream sequence of human Fzd5 or rat Fzd2 were kindly provided by Dr. Ray Habas and Dr. Xi He. The open reading frames were amplified by PCR, cloned into pcDNA3.1 and subsequently subcloned into the lentiviral vector pLenti6.3 (cat. no. K5315-20, Invitrogen). The following primers were used for PCR: forward primer (Fzd5 signal peptide): 5′ CAC CAT GGC TCG GCC TGA 3′; reverse primer, Fzd2: 5′ CCT CTA GAC CGC TTC ACA 3′; reverse primer, Fzd5: 5′ GCT CTA CAC GTG CGA CAG 3′. The Super8XTOPFlash reporter construct and pBARLS lentiviral reporter expression system containing 12 tandem repeats of TCF binding sites were kindly provided by Dr. Randall T. Moon [46,47].

2.3. Transfection and transduction

HEK293, C57MG and L cells were transiently transfected with the Super8XTOPFlash reporter, using lipofectamine (cat. no.11668-019, Invitrogen) for HEK293 cells, PolyJet™ transfection reagent (cat. no. SL100688, SignaGen Laboratories, Ijamsville, MD) for C57MG cells and Fugene (cat. no. 11815091001, Roche, Indianapolis, IN) for L cells, all according to the manufacturer's protocols.

To stably express a β-catenin reporter in L cells, we used the lentiviral vector pBARLS that was packaged in HEK293T cells. The HEK293T cells were transfected with 6 μg pBARLS, along with 1.5 μg pMD2C (plasmid 12259, Addgene) and 4.5 μg psPAX2 (plasmid 12260, Addgene) packaging vectors in a 10 cm plate with 36 μl Fugene following the manufacturer's protocol. After 48 h the virus-containing supernatants were collected, clarified using a 0.45 μm syringe filter and concentrated 20-fold by centrifugation (3500 *g*

for 15 min at 4 °C) using Amicon Ultra centrifugal filter units (cat. no. UFC800324, Millipore, Cork, Ireland). A subconfluent (25–30%) monolayer of L cells was incubated with concentrated virus (500 μl) diluted in 10 ml of MEM, and Polybrene (8 μg/ml) (cat. no. MCPROTO055, Millipore, Billerica, MA) was added to enhance the transduction efficiency. After 24 h, culture medium was changed to standard L cell medium (see above), and 24 h later cells were split (1:5 ratio) and cultured with 10 μg/ml puromycin (cat. no. P8833, Sigma, St. Louis, MO) for 10 days to obtain stable transfectants. The above protocol was also used to generate virus concentrate containing pLenti6.3/HA-humanFzd5 or pLenti6.3/HA-ratFzd2 or pLenti6.3/V5-GW/lacZ empty vector for the transduction of L cells stably expressing the pBARLS luciferase reporter. After 48 h, cells were treated with 2 μg/ml blasticidin (cat. no. 203350, Millipore) for 2 weeks to obtain cell lines stably expressing both the reporter and the appropriate Fzd or empty vector construct.

2.4. Luciferase reporter assay

After cells were grown to confluence in serum-containing medium, they were switched to serum-free medium for 6 h, and then incubated with the indicated proteins overnight. Cell lysates were prepared with reporter lysis buffer from the Luciferase Assay System kit (cat. no. E1501, Promega, Madison, WI) and clarified by centrifugation at 20,800 *g* for 10 min at 4 °C. The supernatant was analyzed according to the manufacturer's protocol using a microplate luminometer (Microlumat Plus Microplate luminometer LB96V, EG & G Berthold, Bad Wildbad, Germany). Luciferase activity was normalized to the protein concentration of cell lysate as determined with the Bio-Rad protein assay kit (cat. no. 500-0006, Hercules, CA). Experiments were performed with biological triplicates for each treatment group.

2.5. Antibodies, recombinant proteins and chemicals

The HA-Fzd proteins were detected with a rat HAmonoclonal antibody 3F10/HRP conjugate (cat. no. 13184200, Roche). Mouse anti β-catenin (cat. no.610154) was purchased from BD Biosciences (San Jose, CA), and anti-heat shock protein-70 (HSP70, sc-7298) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Myc (cat. no. P/N 46-0603), Alexa Fluor 488 conjugated goat anti-mouse secondary antibody (cat. no. A11001) and Alexa Fluor 568 phalloidin (cat. no. A-21124) were from Invitrogen. Anti-mouse IgG (cat. no. NA931V) and anti-rat IgG (cat. no. NA935V) secondary antibodies conjugated with HRP were from GE Healthcare (Buckinghamshire, UK). DAPI (cat. no. D9542) was from Sigma-Aldrich (St. Louis, MO). Recombinant mouse Wnt3a (cat. no.1324-WN) and Wnt3a antibody (cat no. MAB1324) were from R&D Systems (Minneapolis, MN). Recombinant sFRP1 and the Myc/His-tagged derivatives, sFRP1-M/H and sFRP1-Δ1-M/H were prepared as described previously [18], as was rat sFRP2 [48] and CRD_{sFRP1} [44]. Bovine serum albumin (BSA) (cat. no. 82-047-3) was from Millipore.

2.6. Immunoblotting

Western blot analysis was performed essentially as previously described [49]. In brief, cells were washed with phosphate-buffered saline (PBS) and treated with lysis buffer (50 mM HEPES, pH7.5, 50 mM NaCl, 1% Triton X-100, 5 mM NaF, 6.7 mM $Na_2P_2O_7$, 1mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation and protein concentration was determined with Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Thirty microgram aliquots were resuspended in 2 × SDS sample buffer and incubated 10 min at 37 °C for Fzd detection or boiled 10 min for analysis of other proteins. Samples were resolved by SDS-PAGE with 10% or 4–20% polyacrylamide Tris–glycine gels (Criterion Precast Gel; Bio-Rad Laboratories) and transferred to Immobilon-P membrane (Millipore). Membranes were

blocked for 30 min with 5% milk and incubated with the indicated primary and corresponding secondary antibodies. Proteins were visualized with SuperSignal Femto chemiluminescent reagents (Thermo Scientific) and BioMax film (Eastman Kodak Co., Rochester, NY) [18].

2.7. β-Catenin stabilization assay

After growing L cell transfectants to ~80% confluency in serum-containing medium, cells were incubated for 6 h in serum-free MEM media and then treated for 3 h with recombinant Wnt3a (2.5 nM) and different concentrations of sFRP1. Following a wash with PBS, they were lysed and processed for SDS-PAGE and immunoblotting as described above.

2.8. Co-immunoprecipitation

Purified sFRP1-M/H (40 nM) and sFRP1-Δ1-M/H (70 nM)were pre-incubated with 5 nM Wnt3a in 100 μl lysis buffer for 10 min at room temperature. Then Myc antibody (1 μg) was added and samples were incubated overnight at 4 °C in a rotary shaker. After adjusting the volume to 500 μl with lysis buffer, 50 μl of 50% protein G-Sepharose slurry (Amersham Pharmacia Biotech) was added and samples were incubated for 1 h at 4 °C in a rotary shaker. Following centrifugation, samples were washed three times with 1 ml of lysis buffer and final pellets were resuspended in 2 × SDS sample buffer for immunoblotting with anti-Wnt3a (1 μg/ml) or anti-Myc (1:2500) antibody and the corresponding secondary antibody.

2.9. Fluorescence microscopy

Cells grown on glass coverslips were fixed with 3.5% formaldehyde for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, blocked with 1% BSA in PBS for 30 min and incubated with β-catenin antibody diluted 1:1000 in 1% BSA/PBS for 1 h. Samples were stained with Alexa Fluor 488-labeled goat anti-mouse antibody (1:1000), Alexa Fluor 568 phalloidin and DAPI for 1 h. All of these steps were performed at room temperature. Images were obtained with a Leica DM IRB fluorescent microscope using a 63× objective lens (Leica, Germany) and a SPOT camera (Diagnostic Instruments, Inc. Sterling Heights, MI). The images were processed with Adobe Photoshop CS3 (Adobe Inc., San Jose, CA).

2.10. RNA isolation, reverse transcription and quantitative PCR

C57MG cells were seeded in 6-well plates (2.5×10^5 per well) and cultured for 24 h in growth medium. After incubation for 1 h in DMEM without supplements, they were treated overnight with Wnt3a (2.5 nM) alone or in combination with sFRP1 (3 or 300 nM) or CRD_{sFRP1} (10 or 100 nM). Total RNA was isolated using the RNeasy kit (cat. no.74104) from Qiagen (Gaithersburg, MD) according to the manufacturer's protocol. To remove contaminating genomic DNA, RNA was treated with DNase I (cat. no. 18068-015, Invitrogen) for 15 min at room temperature followed by incubation with 2.5 mM EDTA for 10 min at 65 °C.

Reverse transcription was carried out with 2 μg RNA and $Oligo(dT)_{20}$ primer using Super Script III First-Strand Synthesis System for RT-PCR (cat. no. 18080-051, Invitrogen) according to themanufacturer's protocol. Quantitative PCR reactions were performed with Brilliant SYBR Green QPCR Master mix (cat. no. 600548, Agilent Technologies, La Jolla, CA) andMx3005P QPCR instrument from Agilent Technologies. Measurement of β-actin transcript levels using MxPro software for each cDNA sample served as an internal control to normalize the values obtained for Axin2 transcript. Primers and qPCR reaction conditions were previously described [50].

2.11. Statistical analysis

The significance of differences in data was determined with Student's *t*-test, except for the quantitative analysis of nuclear β-catenin staining, which was determined with Fisher's exact test. The differences were considered to be significant when P value was less than 0.05.

3. Results

3.1. sFRP1 effects on Wnt3a/β-catenin signaling vary with dose and cellular context

To evaluate the impact of recombinant sFRP1 on the β-catenin pathway, we used a sensitive and quantitative reporter assay in which the expression of luciferase was under the control of a promoter containing multiple tandem repeats of a TCF binding motif [45–47]. Stabilization of β-catenin results in the increased expression of luciferase, which is detected by measuring the intensity of light generated from a reaction catalyzed by luciferase. Treatment of HEK293/STF cells with sFRP1 alone did not affect luciferase expression, while Wnt3a (2.5 nM) stimulated a strong increase in reporter activity (Fig. 1A). When cells were incubated with a combination of Wnt3a and low concentrations of sFRP1 (1–10 nM), the reporter activity was 3–5 times greater than that seen with Wnt3a alone. However, at higher sFRP1 concentrations (100 and 300 nM), activity was markedly reduced (Fig. 1A). Qualitatively similar results were obtained with parental HEK293 cells transiently transfected with a Super8XTOPFlash reporter (data not shown). Experiments performed with the mouse mammary epithelial line C57MGyielded comparable data, although the magnitude of inhibition was less in C57MG cells treated with 300 nM sFRP1 (Fig. 1B). In contrast, at corresponding concentrations sFRP1 only antagonized Wnt3a activity in L929 fibroblasts (L cells) (Fig. 1C). This differential pattern of sFRP1 activity also was observed when HEK293/STF and L cells were treated with a wide range of Wnt3a concentrations (Fig. 1D and E). These findings demonstrated that the same purified sFRP1 protein could either potentiate or inhibit Wnt3a/β-catenin signaling, depending on the sFRP1 dose and cell line used in the assay.

3.2. Fzd5, but not Fzd2, enables sFRP1 stimulation of Wnt3a activity in L cells

We hypothesized that the nature of the response to sFRP1 was dependent on the particular Fzds expressed by the responding cells. To test this idea, we introduced HA epitope-tagged Fzd2 or Fzd5 into L cells and monitored the dose-dependent effect of sFRP1 in the reporter assay. We expected that cells expressing Fzd5 might facilitate the potentiation of Wnt3a activity by sFRP1, particularly at low sFRP1 concentrations, because this pattern had been observed in S2 cells engineered to express *Drosophila* Fz2 [18], which is closely related to mammalian Fzd5 but not mammalian Fzd2 [51]. As a control, cells also were stably transfected with the backbone lentiviral vector. Cells containing the empty vector (L/eV) behaved like parental L cells, showing a strong dose-dependent inhibitory effect of sFRP1 (Fig. 2A). The L/Fzd2 and L/Fzd5 transfectants contained similar amounts of epitope-tagged receptor (Fig. 2B, western blot analysis), and the stimulatory effect of Wnt3a (2.5 nM) in the reporter assay was enhanced in both lines relative to L/eV cells (Fig. 2A), reflecting the effect of ectopically expressed Wnt receptor. While sFRP1 again exhibited a dose-dependent inhibition of Wnt3a activity in L/Fzd2 cells, it augmented Wnt3a activity in L/Fzd5 cells (Fig. 2A). Consistent with these results, sFRP1 decreased the Wnt3a-dependent accumulation of β-catenin in L/eV whole cell lysates and nuclei, while β-catenin levels were increased in L/Fzd5 lysates and prominent in nuclei of L/Fzd5 cells treated with Wnt3a and sFRP1 (Fig. 2C–E). This suggested that Fzd5, and not Fzd2, mediated aWnt3a-dependent potentiating effect of sFRP1.

3.3. sFRP2 enhances Wnt3a/β-catenin signaling in HEK293/STF and L/Fzd5 cells

Previous reports have claimed that sFRP2 either enhances or inhibits Wnt3a/β-catenin signaling [35,36,52]. We included sFRP2 in our study to compare the effect of dose and cellular context on its activity relative to what we observed with sFRP1. Like sFRP1, sFRP2 potentiated Wnt3a activity in HEK293/STF cells at a low concentration (3 nM). However, in contrast to sFRP1, it also stimulated reporter activity at high concentration (300 nM) (Fig. 3A). sFRP2 also enhanced Wnt3a activity in C57MG cells, with a dose–response resembling that of sFRP1 (Fig. 3B). On the other hand, sFRP1 and sFRP2 displayed similar patterns of inhibitory activity in L/eV cells and stimulatory activity in L/Fzd5 cells (Fig. 3C). These results demonstrated that sFRP2 had context-specific effects on Wnt3a/β-catenin signaling, and indicated that, as for sFRP1, Fzd5 can mediate aWnt3a-dependent potentiating effect of sFRP2.

3.4. Role of CRD_{sFRP1} in the regulation of Wnt3a/β-catenin signaling

To further investigate the mechanisms responsible for sFRP1 regulation of Wnt3a/β-catenin signaling, we examined the activity of its netrin domain and CRD_{sFRP1} in various assays. Results with the netrin domain were variable and therefore inconclusive. Our findings with CRD_{sFRP1} consistently demonstrated that it mimicked the potentiating effects of full-length sFRP1. It enhanced Wnt3a-dependent reporter activity in HEK293/STF cells over a wide concentration range, and did not inhibit activity at doses as high as 2000 nM (Fig. 4A). Both CRD_{sFRP1} and sFRP1 increased theWnt3a-induced expression of the prototypical β-catenin target gene, Axin2, in C57MG cells, although the effect of CRD_{sFRP1} was not as strong at the concentrations tested (Fig. 4B). Interestingly, CRD_{sFRP1} showed little ability to bindWnt3a in comparison to sFRP1 in a co-immunoprecipitation assay (Fig. 5). We conclude that CRD_{sFRP1} potentiates rather than inhibits Wnt3a/β-catenin signaling, and probably acts via a mechanism that does not involve binding to Wnt3a.

4. Discussion

This report provides new evidence that sFRPs either enhance or inhibit Wnt/β-catenin signaling, depending on their concentration and cellular context. This reinforces and clarifies information from previous studies. The biphasic activity of sFRP1 observed withWnt3a in HEK293 cells mirrors the pattern we previously described for sFRP1 and Wingless in *Drosophila* S2 cells [18], and differs sharply from the response seen here and by others with Wnt3a in L cells. The potentiating effect of sFRP1 on Wnt3a activity in C57MG cells demonstrates that its enhancement of Wnt/β-catenin signaling is not likely to be a rare event, rather it represents the primary response of various cells to nanomolar concentrations of sFRP1. A similar picture was seen with sFRP2: stimulation of Wnt3a activity in HEK293 and C57MG cells but inhibition in L cells. The ectopic expression of Fzd5 in L cells altered their response to sFRP1 and sFRP2, enabling both proteins to increase Wnt3a/β-catenin signaling at concentrations that previously inhibited the pathway. This implied that the profile of Wnt receptor expression could determine the impact of sFRPs on β-catenin transcriptional activity.

Initial research suggested that sFRPs functioned as Wnt antagonists by binding to Wnt proteins via their CRD, analogous to Fzds, and thereby preventing access to the cell surface receptors [12,16]. Subsequent articles indicated that the netrin domain also contributed to the inhibitory activity of sFRP1 [19,53], and was primarily responsible for binding to Wnts [18,19]. Here, we have shown that CRD_{sFRP1} exhibits little avidity forWnt3a, but it increases Wnt3a activity in the β-catenin pathway. This implies that the potentiating effect of CRD_{sFRP1} and sFRP1 does not require Wnt-binding, although the association of sFRP1 with Wnt3a might augment its stimulatory activity, which is evident at lower concentrations

than CRD_{sFRP1}. We propose that the potentiating effect depends on an interaction between CRD_{sFRP1} and CRD_{Fzd}. Instead of displacing Wnt from CRD_{Fzd}, the CRD_{sFRP1}-CRD_{Fzd} interaction likely promotes Wnt/CRD_{Fzd} binding via an allosteric mechanism, or perhaps alters the intracellular processing of Fzds. Technical difficulties hampered our attempts to evaluate the effect of CRD_{sFRP1} onWnt3a/CRD_{Fzd} binding in cell-free and cell-based co-immunoprecipitation assays. It would be of interest to examine the mutual interaction of these components, perhaps combining them at higher concentrations for chromatographic and X-ray structural analysis, as recently described for *Xenopus* Wnt8 and mouse CRD_{Fzd8} [54].

Besides sFRP interactions with Fzds and Wnts that directly regulate the β-catenin pathway, additional mechanisms may contribute to the modulation of the pathway by sFRPs. As previously noted, non-canonical Wnt signaling can inhibit β-catenin transcriptional activity by increasing β-catenin turnover [8,10] or reducing β-catenin/TCF association with DNA [9]. Such mechanisms might be activated by sFRPs alone or in combination with Wnts via binding to particular Fzds. Both Wnts and sFRPs associate with proteoglycans that control their distribution and perhaps function as Wnt co-receptors [6,13,18]. Interactions with various proteoglycans might facilitate sFRP-dependent Wnt presentation to, or sequestration from, its receptors. The biphasic dose-dependent effects of sFRPs may reflect their ability to form homodimers, which presumably could affect their association with Fzds, Wnts, proteolglycans and perhaps other factors that have an impact on Wnt signaling.

In summary, the present work supports a revisionist view that sFRPs enhance rather than antagonize Wnt signaling in some cellular contexts, though they clearly inhibit the β-catenin pathway in other settings. The CRD_{sFRP1} potentiated Wnt3a/β-catenin signaling, but exhibited little avidity for Wnt protein, implying that CRD_{sFRP1} activity probably depends on interactions with CRD_{Fzd} or other yet to be described mechanisms. Multiple studies suggest that the up-regulation of sFRP expression may be a valid therapeutic modality for cancers in which they are silenced by promoter hypermethylation [55,56]. The risk-benefit of such interventions may warrant such treatment for neoplasia, though one should be mindful that increasing sFRP levels probably would enhance Wnt/β-catenin signaling in some tissues.

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Abbreviations

BSA	bovine serum albumin
CRD	cysteine-rich domain
Fzd	Frizzled
PBS	phosphate-buffered saline
sFRP	secreted Frizzled-related protein

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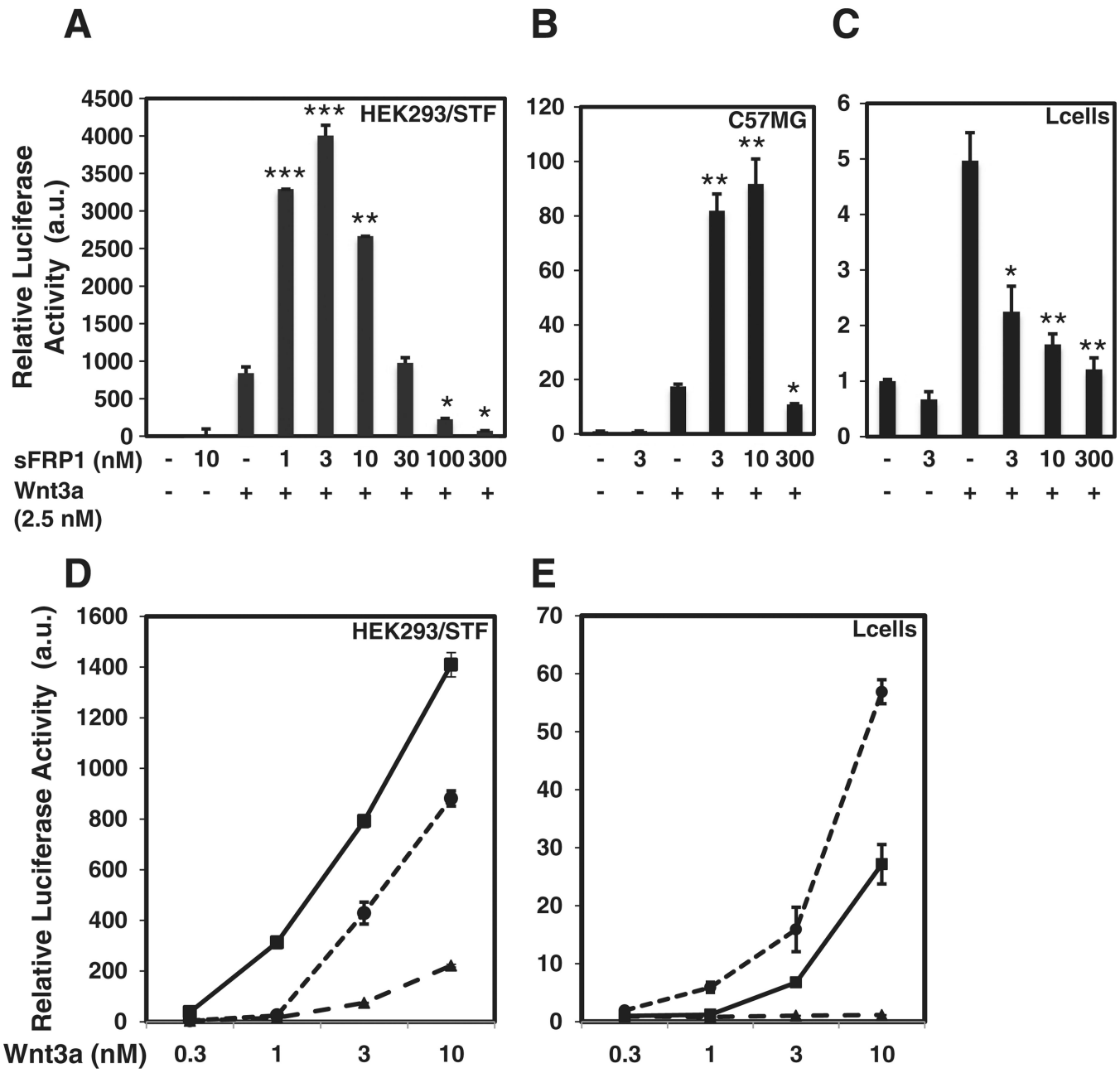

Fig. 1.

sFRP1 modulation of Wnt3a/β-catenin promoter reporter activity varies with cell context. (A) HEK293 cells stably expressing a SuperTopFlash reporter, and (B) C57MG and (C) L cells transiently expressing the Super8XTopFlash reporter were incubated overnight with the indicated concentrations of sFRP1 in the presence or absence of Wnt3a (2.5 nM). In the absence of Wnt3a, BSA was added as a vehicle control. The luminescence of cell lysates was measured and normalized to total cellular protein concentration. Relative luminescence was expressed in arbitrary units (a.u.) with 1 a.u. = luminescence observed in the absence of Wnt3a and sFRP1. Results are expressed as the mean ± S.D. of biological triplicate samples from a representative experiment (at least 3 experiments were performed with each cell line). Statistical significance was determined with Student's *t*-test, comparing results to those obtained in the presence of Wnt3a alone. *P < 0.05; **P < 0.005; ***P < 0.0005. (D)

HEK293/STF and (E) L cells stably expressing pBARLS were incubated with the indicated concentrations of Wnt3a in the absence of sFRP1 (circles) or the presence of 3 nMsFRP1 (squares) or 300 nMsFRP1 (triangles). Results from representative experiments (3 performed with each cell line) were obtained and expressed as described in (A–C).

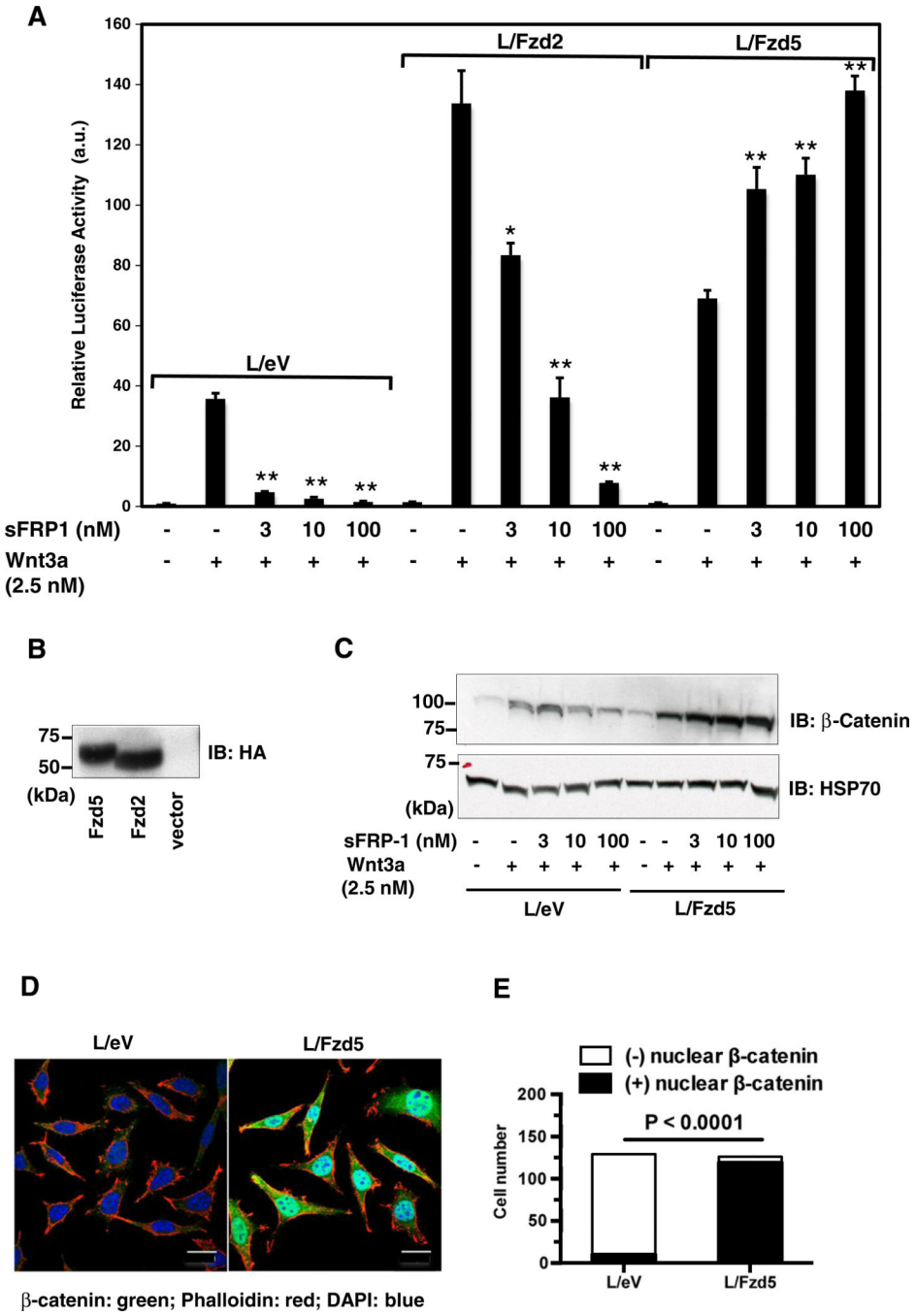

Fig. 2.

sFRP1 regulation of Wnt3a/β-catenin signaling in L cells expressing HA tagged Fzd2 vs. Fzd5. (A) Reporter assay. L cells stably expressing the pBARLS reporter were stably transfected with empty lentiviral vector (L/eV) or vector encoding HA-tagged Fzd2 (L/Fzd2) or Fzd5 (L/Fzd5). Cells were incubated overnight with the indicated concentrations of sFRP1 andWnt3a. Relative luminescence was measured as described in the legend in Fig. 1. Results are expressed as the mean ± S.D. of biological triplicate samples from a representative experiment (at least 3 experiments were performed with each cell line). Statistical significance was determined with Student's *t*-test, comparing results to those

obtained in the presence of Wnt3a alone. *P < 0.05; **P < 0.005. (B) Western blot analysis. Equivalent amounts (30 μg/lane) of cell lysates from the three L cell transfectants were immunoblotted with antibody to the HA epitope. The positions of molecular mass markers are indicated. (C) β-catenin stabilization assay. L/eV and L/Fzd5 cells were treated for 3 h with the indicated concentrations of sFRP1 and/orWnt3a. In the absence of Wnt3a, BSA was added as a vehicle control. Equivalent amounts of cell lysates (30 μg/lane) were immunoblotted for β-catenin and HSP70, the latter serving as a loading control. (D) Intracellular distribution of β-catenin. L/eV and L/Fzd5 cells were treated withWnt3a and 300 nMsFRP1 for 3 h. Cells were fixed, permeabilized and stained with β-catenin antibody, phalloidin to visualize polymerized actin and DAPI to detect nuclear β-catenin by confocal microscopy. Bars = 20 μm. (E) Quantitative analysis of experiments illustrated in (D), indicating the number of cells surveyed that displayed nuclear β-catenin.

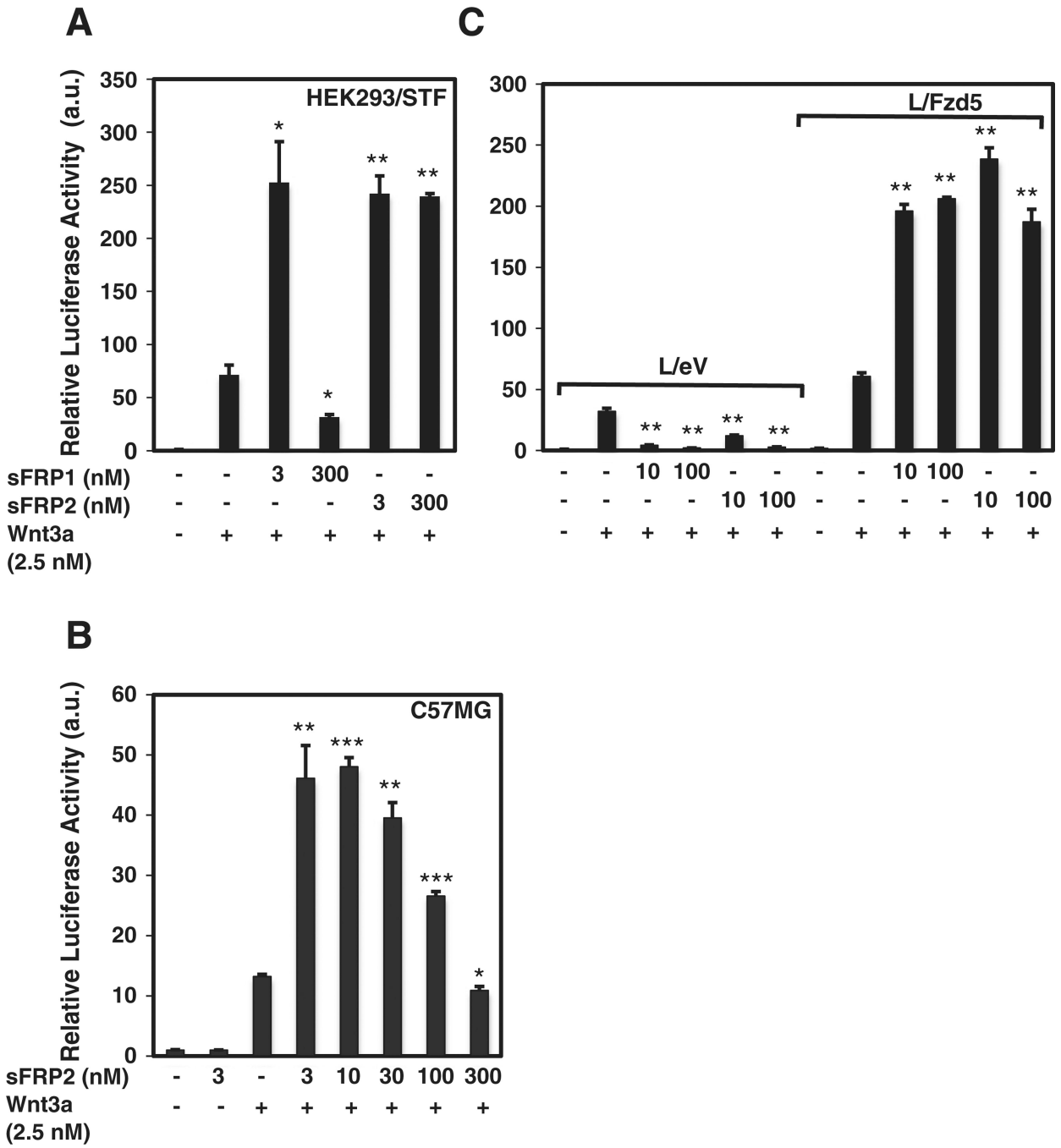

Fig. 3.

sFRP1 and sFRP2 regulation of Wnt3a/β-catenin-dependent reporter activity in HEK293/STF, C57MG and L/Fzd5 cells. (A) HEK293/STF, (B) C57MG and (C) L/eV and L/Fzd5 cells were incubated overnight with the indicated concentrations of sFRP1 or sFRP2 andWnt3a. Relative luminescence was measured and statistical significance was determined with Student's *t*-test, comparing results to those obtained in the presence of Wnt3a alone. *P < 0.01; **P < 0.001.

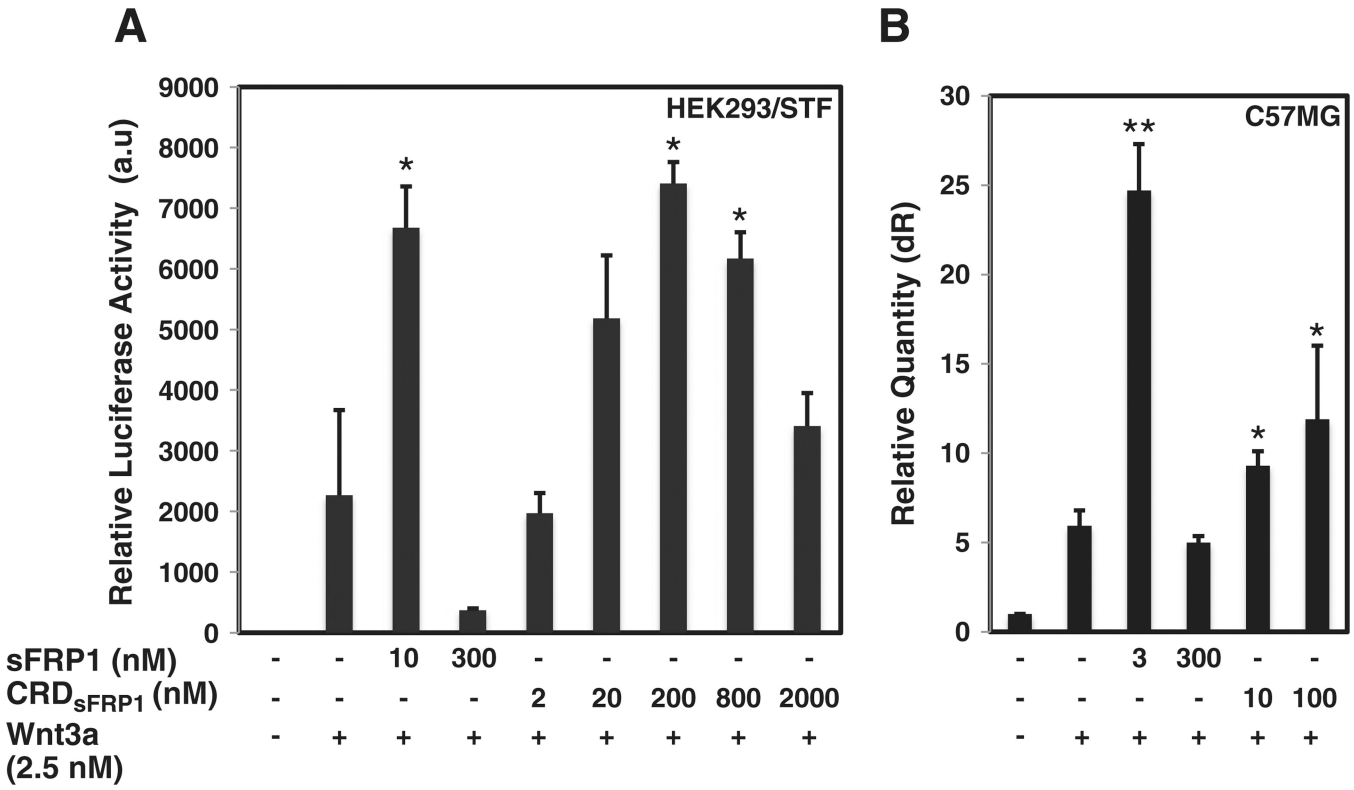

Fig. 4.

Comparison of CRD_{sFRP1} and sFRP1 effects onWnt3a/β-catenin-dependent transcription. (A)HEK293/STF cells were treated overnight with the indicated concentrations of CRD_{sFRP1} or sFRP1 and Wnt3a, and subsequently processed for measurement of relative luminescence as previously described. (B) C57MG cells were treated overnight with the indicated concentrations of sFRP1 or CRD_{sFRP1} andWnt3a, followed by quantitative PCR analysis of *Axin2* and β-*actin*. Results in (A) and (B) are expressed as the mean ± S.D. of biological triplicate samples from a representative experiment [3 experiments were performed for (A) and 2 for (B)]. Statistical significance of data in (A) and (B) was determined with Student's *t*-test, comparing results to those obtained in the presence of Wnt3a alone. *P < 0.05; **P < 0.005.

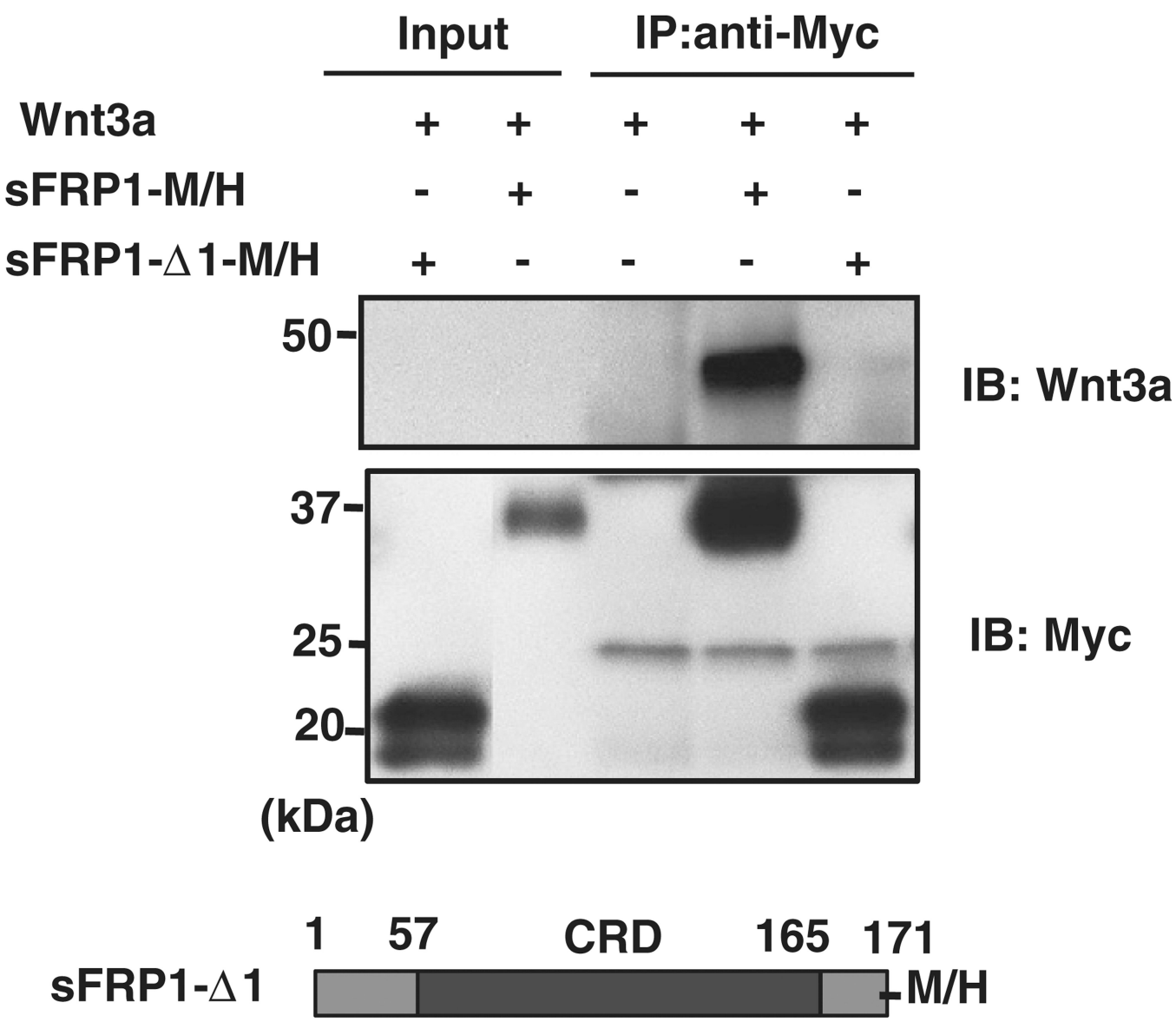

Fig. 5.

Co-immunoprecipitation of Wnt3a with Myc/His-tagged sFRP1 and CRD_{sFRP1} derivatives. Wnt3a was incubated with epitope-tagged full-length sFRP1 or sFRP1-Δ1, an analog of CRD_{sFRP1}, and subsequently co-immunoprecipitated with Myc antibody. Pelleted proteins were immunoblotted with antibodies toWn3a (upper panel) and Myc (lower panel). The lower panel also includes aliquots corresponding to 12% of sFRP1 and 29% of sFRP1-Δ1 used in the co-immunoprecipitation experiment. Below the panels is a schematic diagram of sFRP1-Δ1 highlighting the amino acid residues that define its boundaries.