The Human Frizzled 6 (HFz6) Acts as a Negative Regulator of the Canonical Wnt-β-Catenin Signaling Cascade

Previously we have cloned the human Frizzled 1 (HFz1) and shown that it transmits the Wnt-3a-induced canonical pathway. We also cloned the human Frizzled 6 (HFz6) and show in the present study that, as opposed to HFz1, HFz6 did not activate the canonical Wnt pathway following exposure to various Wnts, whether belonging to the Wnt-1 or to the Wnt-5a group. Moreover we show that HFz6 repressed Wnt-3a-induced canonical signaling when co-expressed with HFz1. HFz6 repressed the canonical Wnt cascade activated also by various Wnt signaling intracellular mediators such as Dishevelled-1, a stabilized β-catenin(S33Y) mutant, and LiCl-mediated repression of glycogen synthase kinase-3β activity. Removal of HFz6 N’- or C-terminal sequences abolished HFz6 repressive activity. As the HFz6 repressive effect was not associated with a decrease in the level of β-catenin, it is suggested that HFz6 does not affect β-catenin stabilization, implying that HFz6 transmits a repressive signaling that cross-talks with and inhibits the canonical Wnt pathway downstream of β-catenin destruction complex. HFz6 did not affect the level of nuclear T-cell factor 4 (TCF4) nor did it affect β-catenin-TCF4 complex formation. However, electrophoretic mobility shift assays indicated that HFz6 repressed the binding of TCF/lymphoid enhancer factor transcription factors to target DNA. Moreover we present data suggesting that HFz6 activates the transforming growth factor-β-activated kinase-NEMO-like kinase pathway that blocks TCF/lymphoid enhancer factor binding to target promoters, thereby inhibiting the ability of β-catenin to activate transcription of Wnt target genes.

Wnt proteins are cysteine-rich secreted glycoproteins serving as extracellular signaling molecules, which play significant roles in normal and pathological developmental processes (1). Based on their biological activities, vertebrate Wnts have been classified into two functional groups with separate downstream signaling pathways. While members of the Wnt-1 class (such as Wnt-1, -2, -3, and -8) commonly stimulate the canonical Wnt/β-catenin pathway that controls cell fate and proliferation, members of the Wnt-5a class (such as Wnt-4, -5a, and -11) stimulate the noncanonical Wnt pathways such as the Wnt/Ca2+ (2–5) and the RhoA/C-Jun N-terminal kinase (for a review, see Ref. 6) signaling cascades. The canonical Wnt signaling is initiated following binding of Wnt proteins to Frizzleds, which interact with members of the low density lipoprotein receptor-related proteins, LRP5 and LRP6, recently recognized as Wnts co-receptors (7–9). This interaction leads to activation of Dishevelled-1 (Dvl-1) proteins, which withhold the glycogen synthase kinase-3β (GSK-3β) activity. In the absence of Wnt, β-catenin resides in a complex with various components such as GSK-3β, Axin, and adenomatous polyposis coli (APC), which together comprise the destruction complex for β-catenin degradation. Inhibition of GSK-3β activity by activated Dvl-1 leads to the stabilization of β-catenin (for a review, see Ref. 10). Stabilized β-catenin accumulates in the cytosol and then translocates to the nucleus where it interacts with members of the lymphoid enhancer factor-1 (LEF)/T-cell factor (TCF) transcription factor family. The TCF/LEF-β-catenin transcription complex activates expression of Wnt target genes, which trigger cell proliferation, oncogenic transformation, and inhibition of apoptosis (for reviews, see Refs. 11 and 12).

Wnts bind to members of the Frizzled receptor family, and the specific transmitted pathway is governed by the formed Wnt-Frizzled complex. Thus, in Xenopus it was reported that members of the Frizzled family such as Rfz2, Mfz3, Mfz4, and Mfz6, but not Rfz1, Mfz7, and Mfz8, stimulate the Wnt/Ca2+ signaling cascade and promote ventral cell fate rather than inducing axis duplication via the canonical Wnt/β-catenin pathway (13).

The canonical and the noncanonical Wnt signaling cascades were reported to exhibit antagonistic interactions, and several mechanisms have been proposed to explain this antagonism (for reviews, see Refs. 14 and 15). One of these mechanisms that was exerted by Wnt-5a and the rat Frizzled 2 (4, 13, 16) involved the activation of Nemo-like kinase (NLK), via TAK1, which phosphorylates TCF/LEF transcription factors and inhibits their DNA binding activity (4, 13, 16, 17).

The multimember Frizzled family comprises proteins with a large cysteine-rich extracellular domain (CRD), a seven-transmembrane-spanning domain, and a cytoplasmic tail that significantly differs in length and sequence similarity.2 Previously

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1 The abbreviations used are: Dvl, Dishevelled; APC, adenomatous polyposis coli; CaMKII, calmodulin-dependent protein kinase II; CRD, cysteine-rich domain; EMSA, electrophoretic mobility shift assay; GSK-3β, glycogen synthase kinase-3β; HA, hemagglutinin; HFz, human Frizzled; Mfz, mouse Frizzled; Rfz, rat Frizzled; Dfz, Drosophila Frizzled; LEF, lymphoid enhancer factor; TCF, T-cell factor; Luc, luciferase; NLK, NEMO-like kinase; nt, nucleotide(s); siRNA, short interfering RNA; SSC, saline-sodium citrate; TAK1, transforming growth factor-β-activated kinase; MOPS, 4-morpholinepropanesulfonic acid.

2 See www.stanford.edu/~rnuse/wntwindow.html.
we have molecularly cloned the human Fzd1 (HFz1) and 6 (HFz6) and showed that HFz1 efficiently transduced the Wnt-3a-induced canonical signaling in mammalian cells (19). Here we show that, unlike HFz1, HFz6 did not transduce the canonical Wnt pathway upon exposure to various Wnts. Furthermore our findings showed that enforced expression of HFz6 down-regulated the transmission of Wnt-3a-induced canonical signaling in HFz1. In addition, we present data suggesting that HFz6 represses the canonical Wnt pathway by transmitting an antagonistic signaling cascade. Moreover the results suggest that HFz6 activates the TAK1-NLK pathway, thus blocking TCF transcriptional activity by interfering with the binding of TCF-β-catenin complex to target DNA.

EXPERIMENTAL PROCEDURES

Plasmids—HFz6 CDNA (GenBank™ accession number AF072873), previously cloned by us from a human breast AB589 cDNA library prepared in pCEV27 vector, is described elsewhere (20). It contained a 2.1-kb coding region and a 2.3-kb 3’ untranslated segment. HFz6 open reading frame was found to encode a predicted 706-amino acid protein that was identical to the open reading frame of the HFz6 cDNA cloned later by Tsuchida et al. (21). HFz6 CDNA was subsequently subcloned into pCEV28, a derivative of pCEV27, which harbors an SV40 origin of replication. The HFz6-ΔCTD (203–835) was PCR-amplified and cloned into pDNA3, downstream of and in-frame to a FLAG epitope, as described previously (22). HFz6-ΔCTD was constructed by PCR amplifying the HFz6 coding region, which spans at 946–2401, and inserting it into pFLAG-CMV1 (Sigma) downstream of and in-frame to a FLAG epitope bordered by an upstream signal peptide. HFz6-ΔΔC was subcloned from the full-length HFz6 by BstXI digestion (nt 1343) and religation, which resulted in the removal of the C-terminal 147 amino acids. HFz1 was described previously (19). pSUPER, containing the H1 promoter for expressing short interfering RNAs (siRNAs) was kindly provided by Dr. R. Agami (The Netherlands Cancer Institute, Amsterdam, The Netherlands). pSUPER-hsHFz6, which expresses siRNAs targeting HFz6 mRNA, was constructed by annealing a forward 5‘-GATCCGGCCAGCTCTCGCTCACTGGTAGACTGAGTTGAGAAGATGAGTGTGTTTTGAAA-3’ oligonucleotide with its complementary strand and inserting the double-stranded oligonucleotide stretch into the BglII/HindIII sites of pSUPER. HA-tagged Wnt-1, -3, and -3a (0.1 µg of cell lysates were subjected to Western blot analysis. Luciferase levels were measured using the dual luciferase assay kit (Promega), and the firefly luciferase activity was normalized to Renilla luciferase activity. Data are presented as mean values and S.D. for at least three independent experiments done in duplicate compared with the level of luciferase activity obtained in the presence of empty vector that is presented as 1. 30 µg of cell lysates were subjected to Western analysis using anti-HA antibody. IB, immunoblot.

Northern Analysis—Total RNA was extracted using the TRI Reagent isolation solution (Molecular Research Center Inc.) according to the manufacturer’s instructions. RNA (20 µg) was resolved on a 1.2% agarose gel containing 6% formaldehyde and transferred to nylon membrane (Hybond™-N, Amersham Biosciences). The membrane was pre-hybridized in hybridization solution (50% formamide, 5× SSC, 4°C Denhardt’s solution, 0.5% SDS, 0.1 mg/ml salmon sperm DNA) for 4 h at 42°C and then hybridized for 18 h with the full-length coding region of HFz6 does not transmit the canonical Wnt signaling pathway. 293T cells were transiently transfected with empty vector, or NFκB or NFκB1 (1 µg each), HA-tagged Wnt-1, -3, and -3a (0.1 µg). TCF/Luc reporters (1 µg), and β-galactosidase (0.1 µg). 48 h after transfection, cell lysates were measured for luciferase and β-galactosidase activities. Data are presented as mean values and S.D. for at least three independent experiments done in duplicate compared with the level of luciferase activity obtained in the presence of empty vector that is presented as 1. Stably transfected SW480 and HCT116 cells were prepared by selection in the presence of 0.5 mg/ml G418 (Calbiochem). KN-93 was obtained from Calbiochem.

Subcellular Fractionation—Cytoplasmic and nuclear fractions were prepared as described previously (26). 293T cells were washed in ice-cold phosphate-buffered saline and resuspended in cold buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM EGTA, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and Complete EDTA-free protease inhibitors (Roche Diagnostics). Resuspended cells were lysed in 0.5% Nonidet P-40 for 15 min followed by centrifugation at 1,000 × g for 5 min. Isolation of nuclear fractions, the nuclei were harvested by resuspension in ice-cold buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM dithiothreitol) followed by centrifugation at 17,000 × g for 15 min at 4°C.

Immunoprecipitation and Western Blot Analysis—Total cell lysates were prepared by solubilization in lysing buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.2% Nonidet P-40). Nuclear and cytosolic fractions were prepared as described above. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes, and after blocking with 5% low fat milk, filters were incubated with the specific primary antibody. Membranes were washed in 0.001% Tween 20 in phosphate-buffered saline and incubated for 45 min with a secondary antibody. After washing in Tween/phosphate-buffered saline, membranes were subjected to enhanced chemiluminescence (ECL) detection analysis (Amersham Biosciences) using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). For co-immunoprecipitation analysis, cell lysates were solubilized in lysing buffer (see above), and extracts were clarified by centrifugation at 12,000 × g for 30 min at 4°C. 4 µg of cell lysates were subjected to Western analysis using anti-HA antibody. IB, immunoblot.
92 of total cell lysates were incubated with the specific antibody for 18 h at 4°C and then incubated, with protein A beads for 2 h at 4°C. Beads were collected by centrifugation, washed three times in lysis buffer, and analyzed by SDS-PAGE followed by detection with each specific antibody. Anti-β-catenin, -c-Myc, and -Cyclin E monoclonal antibodies, anti-Cyclin D1, -β-actin, and -HA polyclonal antibodies, and anti-goat, -rabbit, and -mouse IgG horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Anti-FLAG M2 monoclonal antibody and anti-FLAG M2-agarose affinity gel were purchased from Sigma.

Electrophoretic Mobility Shift Assay (EMSA)—The TCF probe (wild type), spanning three potential TCF/LEF binding sites, was a double-stranded 40-nucleotide oligomer prepared by annealing a forward 5'-GGGGGCCTTTGATCCCTTTGATCCCTTTGATCCCTTTGATC-3' oligonucleotide and its reverse form 5'-GGGGGATCAAAGGGATCAAAGGATCAAAGGATCAAAGGATCAAAGGATCAAAGG-3'. The mutant probe was prepared by annealing the mutated forward 5'-GGGGGCTTTGATCCCTTTGATCCCTTTGATCCCTTTGATC-3' and its reverse 5'-GGGGGATCAAAGGGATCAAAGGATCAAAGGATCAAAGGATCAAAGGATCAAAGGATCAAAGG-3' oligonucleotides. Nuclear extracts were prepared as described above. Binding reactions were done on ice for 10 min by incubating 6 μg of nuclear lysates and 0.022 pmol of labeled double-stranded wild type or mutant probe in 15 μl of binding buffer (100 mM Tris, pH 8, 300 mM KCl, 25 mM MgCl2, 20% glycerol, and 0.75 μg of poly(dI-dC)). Competition analyses were performed with an excess (1.0 pmol) of unlabeled probe. Supershift analyses were performed by adding 4 μl of specific antibodies to the reaction mixture. Samples were resolved by 5% PAGE in 0.5 TBE (45 mM Tris borate, 1 mM EDTA) buffer, and the radioactive bands were visualized by autoradiography.

In Vitro Kinase Assay—Cells were solubilized in lysing buffer (50 mM β-glycerol phosphate, 1.5 mM EGTA, 1 mM EDTA, 10 mM sodium orthovanadate, 1 mM benzamidine hydrochloride, 1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride). After separating NLK-FLAG from cell lysate using anti-FLAG M2-agarose affinity gel, aliquots of immunoprecipitates were washed twice with lysis buffer, twice with washing buffer (500 mM LiCl, 100 mM Tris, pH 7.6, 0.1% Triton X-100), and twice with kinase buffer (20 mM MOPS, pH 7.6, 2 mM EGTA, 10 mM MgCl2, 1 mM diithiothreitol, 0.1% Triton X-100, 1 mM sodium orthovanadate). Kinase reactions were performed in kinase buffer in a total volume of 30 μl in the presence of 100 μM cold ATP and 5 μCi of [γ-32P]ATP at 30 °C for 30 min. Following the kinase reaction, samples were resolved by SDS-PAGE, and the phosphorylated proteins were visualized by autoradiography.
RESULTS

HFz6 Does Not Transmit the Canonical Wnt Signaling following Exposure to Various Wnts—Previously we have shown that HFz1 transduces the canonical TCF-dependent Wnt signaling pathway following exposure to either Wnt-3a, Wnt-3, Wnt-1, or Wnt-2 both in an autocrine or a paracrine mode (19). Results show whether HFz6 might also serve as a receptor transducing the canonical Wnt pathway, 293T cells were co-transfected with HFz6; Wnt-1,-3, -3a; and TCF/Luc reporters. Data showed that, as opposed to HFz1, HFz6 did not transduce the canonical Wnt signaling in the presence of any of the tested Wnts (Fig. 1). HFz6 did not transduce the canonical Wnt pathway stimulated by Wnt-3a or Wnt-4 (not shown).

HFz6 Antagonizes Wnt-3a Signal Transduction via Dvl1—To investigate whether HFz6 affects the canonical Wnt pathway transduced via Dvl1, 293T cells were co-transfected with HFz6, Wnt-3a, increasing amounts of HFz6 or empty vector, and TCF/Luc reporters. Data showed that HFz6 inhibited Wnt-3a/HFz1-induced transcriptional activation of the TCF/Luc reporter in a dose-dependent manner (Fig. 2A). To verify that the forced expression of HFz6 was specifically responsible for the repression of Wnt-3a/HFz1-induced TCF-dependent signaling, we used the RNA interference approach (27). We constructed pSUPER expressing short interfering HFz6-specific RNAs (HFz6-siRNA), which efficiently reduced the level of HFz6-CRD-FLAG (Fig. 2B). Data showed that co-expressing HFz6-siRNA abolished the repressive activity exhibited by the ectopically expressed HFz6 (Fig. 2C).

HFz6 Inhibits the Canonical Wnt Pathway Switched on by Downstream Wnt Signaling Activators—To gain insight into the mechanism of the HFz6 inhibitory effect on the Wnt canonical pathway, experiments were performed to map the position of HFz6 inhibitory activity along the Wnt cascade. The approach involved the activation of the canonical Wnt pathway by downstream Wnt signaling activators and investigating whether Wnt signaling would be repressed by ectopically expressed HFz6. Overexpression of Dvl1 in a mammalian cell culture was previously shown to activate transcription from a reporter plasmid driven by TCF-responsive elements (28). To determine whether co-expression of HFz6 would inhibit the Dvl1-induced signaling pathway, 293T cells were co-transfected with a Myc-tagged Dvl1 (Dvl1-Myc) and HFz6 or empty vector. Data showed that ectopically expressed HFz6 inhibited Dvl1-induced Wnt signaling (Fig. 3A), albeit the presence of similar amounts of Dvl1 in the presence or absence of HFz6, and that this inhibition was released in the presence of HFz6-siRNA (Fig. 3B). The ability of HFz6 to repress the canonical Wnt signaling that was activated by enforced expression of Dvl1 implied that HFz6 repressed Dvl1-induced canonical Wnt pathway by transmitting an antagonistic signaling cascade and that HFz6-transmitted signaling cross-talks with and inhibits the canonical Wnt pathway downstream of, or in parallel, but dominant to Dvl1.

HFz6 Acts Downstream of β-Catenin Destruction Complex—The canonical Wnt pathway is activated by LiCl, which inhibits GSK-3β activity (29). To narrow down the stage of HFz6 action, experiments were performed to investigate whether HFz6 activity would inhibit LiCl-induced Wnt signaling. Data showed that HFz6 overexpression inhibited LiCl-induced signaling (Fig. 4A), although it had no effect on the level of the endogenous β-catenin. The presence of HFz6-siRNA abolished HFz6 repressive activity (Fig. 4B). These data indicated that HFz6 inhibits Wnt signaling at a step downstream of GSK-3β-activated β-catenin phosphorylation. As HFz6 acts downstream of GSK-3β, it was reasonable to assume that HFz6 would inhibit the Wnt pathway induced by a non-degradable β-catenin mutant. We then used a constitutively active β-catenin(S33Y) mutant, which is insensitive to GSK-3β-mediated β-catenin phosphorylation and proteasomal degradation (30). Data showed that Wnt signaling induced by the stabilized β-catenin was inhibited by overexpressing HFz6 (Fig. 4C), albeit the presence of similar levels of β-catenin, and this repression was released by overexpressing HFz6-siRNA (Fig. 4D). To further substantiate the notion that HFz6 does not act by targeting β-catenin for degradation, we investigated HFz6 effect on TCF-dependent signaling activated by an endogenously stabilized β-catenin. The canonical Wnt signaling is constitutively active in various colon cancer (colorectal cancer)-derived cell lines, such as SW480 or HCT116 in which either APC or β-catenin, respectively, is mutated (31, 32). SW480 and HCT116 cells were transiently transfected with HFz6 or empty vector and TCF/Luc reporters, and the level of Wnt signaling was measured. Data showed that HFz6 repressed the constitutively active Wnt pathway in both SW480 and HCT116 cells (Fig. 4, E and F).
Fig. 4. **HFz6 represses the canonical Wnt signaling downstream of β-catenin destruction complex.** 

**A**, HFz6 represses LiCl-induced signaling. 293T cells were co-transfected with HFz6 or empty vector (1 µg), TCF/Luc reporters (1 µg), and β-galactosidase (0.1 µg). 24 h after transfection, cells were treated with 30 mM LiCl. Luciferase and β-galactosidase levels were measured as described in the legend to Fig. 1. 30 µg of cell lysates were subjected to Western analysis using anti-β-catenin antibody.

**B**, HFz6-siRNA released HFz6-mediated repression of LiCl-induced signaling. 293T cells were co-transfected with HFz6 or empty vector (1 µg), TCF/Luc reporters (1 µg), β-galactosidase (0.1 µg), and either empty pSUPER or HFz6-siRNA (2 µg each). 24 h after transfection, cells were treated with 30 mM LiCl. Luciferase and β-galactosidase levels were measured as described in A. HFz6 represses β-catenin-induced signaling. 293T cells were co-transfected with HFz6 or empty vector (1 µg), TCF/Luc reporters (1 µg), β-galactosidase (0.1 µg), and β-catenin(S33Y) or empty vector (0.1 µg each). Luciferase and β-galactosidase levels were measured. 30 µg of cell lysates were subjected to Western analysis using anti-β-catenin antibody.

**D**, HFz6-siRNA released HFz6-mediated repression of β-catenin-induced signaling. 293T cells were co-transfected with empty vector or HFz6 (1 µg), TCF/Luc reporters (1 µg), β-galactosidase (0.1 μg), β-catenin(S33T) (0.1 µg), and either empty pSUPER or HFz6-siRNA (2 µg each). Luciferase and β-galactosidase levels were measured. **E**, HFz6 represses the constitutively active Wnt signaling in SW480 cell line. SW480 cells were transiently transfected with TCF/Luc (1 µg), Renilla luciferase vector (0.5 µg), and HFz6 or empty vector (1 µg each). 48 h after transfection the luciferase activity was measured, and the luciferase levels were normalized according to Renilla luciferase activity. **F**, HFz6 represses the constitutively active Wnt signaling in HCT116 cell line. HCT116 cells were transfected with HFz6 or empty vector (1 µg each), TCF/Luc (1 µg), and β-galactosidase (0.1 µg). The luciferase and β-galactosidase levels were measured.
HFz6 Represses the Canonical Wnt Cascade

**HFz6 Acts by Transmitting a Repressive Signaling Pathway**—Our data, indicating that HFz6 represses the canonical Wnt pathway induced by intracellular Wnt signaling activators, suggested that HFz6 acts by transmitting a repressive signaling pathway. To confirm this notion we investigated whether the HFz6-mediated repressive effect would be abolished by removal either the N’- or C’-terminal HFz6 sequences. Data showed that LiCl-induced canonical Wnt pathway was not repressed either by HFz6-CRD or by HFz6 mutants that lacked the 147 C’-terminal amino acids (HFz6-ΔC) or the N’-terminal CRD (HFz6-ΔCRD) (Fig. 5). As the level of HFz6-ΔC expression was lower than that displayed by the full-length HFz6, the contribution of HFz6 C’ terminus in transmitting the signal could not be conclusively established.

**HFz6 Inhibits Transcription of TCF Target Genes**—Evidently our experiments investigating the effect of HFz6 on the Wnt canonical pathway used TCF-dependent luciferase reporters harboring three copies of a TCF binding motif (24). To further confirm that HFz6 represses TCF transcriptional activity of Wnt target genes, we investigated the effect of HFz6 on a native TCF-responsive promoter. To this end we used a luciferase reporter in which the expression of luciferase is governed by the promoter of cyclin D1, a Wnt target gene containing a TCF-binding domain within its transcriptional units (33). Data showed that ectopically expressed HFz6 reduced the expression levels of CyclinD1/Luc reporter that was activated in 293T cells by LiCl treatment or by overexpressing β-catenin (Fig. 6, A and B). It has been shown that abolishing the constitutively active Wnt signaling pathway in SW480 cells reduced the augmented levels of c-Myc and Cyclin D1 expression (33, 34). To explore whether the elevated expression of Wnt-responsive genes would be lowered by overexpressing HFz6, the level of expression of the endogenous c-Myc and Cyclin D1 was measured in SW480 and HCT116 cells lines transfected with HFz6. Data showed that SW480 and HCT116 clones, stably transfected with HFz6, expressed lower levels of endogenous c-Myc and Cyclin D1 (Fig. 7, A and B). Taken together, our findings strongly suggest that HFz6 repressive signaling affects β-catenin/TCF-targeted transcription. Interestingly the stably HFz6 transfected SW480 and HCT116 cells, although expressing lower levels of Cyclin D1 and c-Myc, were only slightly delayed in their growth characteristics (data not shown).

**HFz6 Does Not Affect β-Catenin Subcellular Distribution**—As HFz6 affects Wnt signaling at the level of β-catenin/TCF-targeted transcription, we sought to investigate whether HFz6 might affect β-catenin nuclear accumulation. 293T cells were transfected with β-catenin(S33Y) mutant, and the level of both nuclear and cytoplasmic β-catenin was measured. Our data, showing similar levels of the ectopically expressed mutated β-catenin in the nuclear or cytoplasmic fractions of 293T transfectants in the presence or absence of HFz6 (Fig. 8), implied that HFz6 does not act by affecting the subcellular localization of β-catenin.

**HFz6 Does Not Affect β-Catenin/TCF4 Complex Formation**—Transcription of Wnt target genes is dependent on β-catenin/TCF interaction (for a review, see Ref. 35). Experiments were thus performed to investigate whether HFz6 interferes with the ability of β-catenin to interact with TCF4. 293T cells were co-transfected with a Myc-tagged β-catenin(S33Y) and TCF4-HA, and either HFz6 or empty vector. Data showed that the same amounts of β-catenin(S33Y) co-immunoprecipitated with TCF4-HA in the presence or absence of HFz6 (Fig. 9).

**HFz6 Represses TCF/LEF DNA Binding Activity**—As HFz6 repressed TCF-directed transcription, although it did not affect TCF/β-catenin complex formation, we next investigated whether HFz6 would affect the binding of TCF/β-catenin complex to target DNA. Nuclear extracts prepared from 293T cells, transiently co-transfected with a Myc-tagged β-catenin(S33Y) and TCF4-HA, were analyzed by EMSAs using a double-stranded oligomer spanning three TCF/LEF potential binding consensus motifs as a probe. Supershift assays performed using anti-β-catenin, anti-HA, or anti-TCF4 antibody showed that the upper retarded band was supershifted by the tested antibodies, indicating that it represented the TCF4-HA/β-catenin complex bound to target DNA (Fig. 10A). However, the lower band, which was present also in non-transfected 293T cells (not shown), was not supershifted by any of the tested antibodies (Fig. 10A). To identify the unshifted band, EMSA was performed using extracts from non-transfected 293T cells. Supershift assays performed with anti-TCF1, anti-LEF-1, anti-TCF3, or anti-TCF4 antibodies showed that this band was supershifted only by the anti-LEF-1 antibody (not shown), suggesting that this band represented the endogenous LEF-1 bound to target DNA. To investigate whether HFz6 modulates TCF4-β-catenin capacity to bind to target DNA, 293T cells were co-transfected with a Myc-tagged β-catenin(S33Y), TCF4-HA, and either HFz6 or empty vector. Data showed that the ectopically expressed HFz6 reduced the formation of TCF4-β-catenin-containing complexes at the TCF4/LEF DNA binding sites by 57% (Fig. 10B). Moreover HFz6 also decreased the amount of endogenous LEF-1 bound to TCF4/LEF DNA probe by 56%. As the nuclear levels of neither β-catenin nor TCF4-HA were diminished in the presence of ectopically expressed HFz6 (Fig. 10C), our data suggest that the HFz6-mediated repression of TCF-dependent transcriptional activity originated from HFz6 inhibitory effect on TCF/LEF DNA binding activity.

**HFz6 Represses the Canonical Wnt Pathway by Activating the TAK1-NLK Signaling**—It has been reported that CaMKII-mediated activation of the TAK1-NLK pathway resulted in TCF/LEF phosphorylation and that this phosphorylation suppresses TCF/LEF binding to target DNA sequences (17). In addition, Wnt-5a and the rat Frizzled 2 (Rfz2) were shown to

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**Fig. 5.** HFz6 truncated mutants do not display HFz6 repressive activity. 293T cells were co-transfected with either HFz6, HFz6-CRD, HFz6-ΔCRD, or HFz6-ΔC (1 μg each); TCF/Luc reporters (1 μg); and β-galactosidase (0.1 μg). 24 h after transfection, cells were treated with 30 mM LiCl, and the luciferase and β-galactosidase levels were measured as described in Fig. 1. 30 μg of cell lysates were subjected to Western analysis using anti-β-catenin antibody. Right panel, Northern blot. 20 μg of total RNA were subjected to Northern analysis using HFz6 coding region as a probe. 28 S ribosomal RNA stained with ethidium bromide was utilized as an internal control for the amount of total RNA loaded. IB, immunoblot.
induce signaling that activated the CaMKII-mediated TAK1-NLK pathway (4, 13, 16), resulting in TCF/LEF phosphorylation (4, 17). Experiments were thus performed to investigate whether HFz6 would also activate the TAK1-NLK pathway and whether the HFz6 repressive effect might be attributed to HFz6-mediated TAK1-NLK activation. 293T cells were co-transfected with NLK-FLAG and HFz6 or empty vector, and NLK kinase activity was measured by an in vitro kinase assay.

Data showed that in the presence of ectopically expressed HFz6, the level of autophosphorylated NLK was augmented (Fig. 11A). These data suggested that HFz6 stimulates the TAK1-NLK signaling pathway. To further elucidate whether HFz6-mediated repression of the DNA binding capacity of TCF/LEF and of β-catenin-TCF complexes results from HFz6-mediated TAK1-NLK activation, we investigated whether abolishment of CaMKII-mediated TAK1-NLK pathway would block the HFz6 repressive effect on Wnt signaling. 293T cells were co-transfected with β-catenin and either HFz6 or empty vector, and the level of HFz6 repressive activity, in the presence of CaMKII inhibitor KN-93 (36) or in the presence of a dominant

FIG. 6. HFz6 represses the transcriptional activity of Wnt target gene promoters. HFz6 represses LiCl-induced activation of cyclin D1 promoter. 293T cells co-transfected with HFz6 or empty vector (1 μg), CyclinD1/Luc (1 μg), and β-galactosidase (0.1 μg) were either treated with 30 mM LiCl 24 after transfection (A) or co-transfected with β-catenin(S33Y) (0.1 μg) (B). Luciferase and β-galactosidase levels were measured. 30 μg of cell lysates were subjected to Western analysis using anti-β-catenin antibody. IB, immunoblot.

FIG. 7. HFz6 represses the endogenous expression of cyclin D1 and c-myc in SW480 and HCT116 cell lines. 100 μg of cell lysates prepared from SW480 (A) or HCT116 (B) clones stably transfected with empty vector or HFz6 (Clones 1–4) were analyzed by Western blotting for the level of expression of Cyclin D1, c-Myc, or β-actin using specific antibodies. Vec, vector.

FIG. 8. HFz6 does not affect β-catenin subcellular distribution. 293T cells, in 9-cm Petri dishes, were co-transfected with HFz6 or empty vector (9 μg) and β-catenin(S33Y) (1 μg). 30 μg of cytosolic or nuclear fraction were subjected to Western analysis using anti-β-catenin antibody and anti-β-catenin antibody and anti-β-actin or anti-Cyclin E antibody, respectively. IB, immunoblot.

FIG. 9. HFz6 does not affect TCF-4/β-catenin complex formation. 293T cells, in 9-cm Petri dishes, were co-transfected with TCF4-HA (2 μg), β-catenin(S33Y) (2 μg), and either HFz6 or empty vector (6 μg). 4 mg of total cellular protein were immunoprecipitated with anti-HA antibody and analyzed by Western blotting with anti-HA before (left panel, 100 μg of protein/lane) or after (right panel) immunoprecipitation. Following stripping, blots were treated with anti-β-catenin antibody. IB, immunoblot; IP, immunoprecipitation.
HFz6 decreases the binding of TCF-β-catenin complexes to TCF binding sites. A, EMSA was performed using nuclear extracts from 293T cells co-transfected with TCF4-HA (0.5 μg) and β-catenin(S33Y) (0.5 μg) in the absence or presence of anti-HA, anti-β-catenin, or anti-TCF4 antibodies. B, EMSA was performed using nuclear extracts from 293T cells co-transfected with TCF4-HA (0.5 μg), β-catenin(S33Y) (0.5 μg), and HFz6 or empty vector (2.5 μg). Samples were incubated with wild type or mutated probe. Unlabeled wild type probe was used as a competitor. A representative gel is shown (top panel). Densitometric analysis of the retarded bands of β-catenin-TCF4 and of LEF-1, from three independent experiments, is shown (bottom panel). Each bar denotes the mean ± S.D. C, 100 μg of nuclear extracts prepared in A were subjected to Western analysis using anti-HA, anti-TCF4 antibodies, or anti-β-catenin antibody. IB, immunoblot; WT, wild type; MT, mutant.

DISCUSSION

HFz6 lacks a PDZ-binding domain at its C terminus but retains the conserved motif KTXXW (nt 1772–1790) critical for the canonical Wnt signaling (37). However, HFz6 did not transmit Wnt-induced β-catenin-TCF-dependent signaling when exposed to various Wnt ligands, whether belonging to either the Wnt-1 or the Wnt-5a family. Conversely our data showed that HFz6 repressed the canonical Wnt signaling induced by Wnt-3a/HFz1 interaction. This observation is consistent with recent data showing that the mouse Fz6, when fused to Xenopus Wnt-8, did not activate the canonical Wnt pathway (38). We present data suggesting that HFz6 represses the Wnt pathway by transmitting an antagonistic signaling. This conclusion is based on our data showing that HFz6 represses the canonical Wnt pathway induced by various downstream intracellular Wnt signaling activators, such as ectopically expressed Dvl-1, treatment with LiCl, or ectopically expressed stabilized β-catenin mutant. HFz6 also inhibited the constitutively active Wnt pathway in SW480 and HCT116 cells in which the endogenous β-catenin is stabilized. Notably this repressive effect was not exhibited by HFz6 mutants that lacked either the CRD or the HFz6 C’ terminus. Thus, our data suggest that the HFz6 repressive effect is attributable to an HFz6-transmitted signaling that cross-talks with and represses Wnt signaling at a step downstream of the β-catenin destruction complex. β-Catenin nuclear translocation is regulated by various factors such as TCP (39) and APC (40) as well as by signaling cascades such as the androgen receptor-transmitted pathway (41). However, our data showing that HFz6 did not affect the total, the cytoplasmic, or the nuclear levels of β-catenin suggest that the ectopically expressed HFz6 does not act via β-catenin destabilization or through regulating β-catenin intracellular distribution. Moreover these data also exclude the possibility that HFz6 destabilizes β-catenin by activating a GSK-3β-independent Siah-mediated β-catenin proteasomal degradation recently reported to be implicated in regulating β-catenin stability (42, 43). In this respect, HFz6 repressive signaling does not resemble Wnt-5a activity recently shown to promote β-catenin degradation in a Siah2- and APC-dependent but GSK-3β-independent manner (44). The level of Wnt signaling is dependent on β-catenin/TCF interaction (for a review, see Ref. 35). However, our data rule out the possibility that HFz6 interferes with β-catenin/TCF4 interaction. EMSA data showed that despite the same levels of β-catenin and TCF4 in nuclear extracts, the level of TCP/LEF and TCF4-β-catenin binding to DNA was decreased in the presence of ectopically expressed HFz6. This suggests that HFz6 repressive activity is not mediated by modulating nuclear translocation of either β-catenin or TCF transcription factors but by interfering with the binding of TCF/LEF and of TCF-β-catenin complexes to target DNA. Previously it has been reported that Wnt-5a, -4, and -11 (14, 45), members of the Wnt-5a group, interact with Frizzled receptors to activate the noncanonical Wnt signaling cascades. Moreover these Wnts were able to antagonize canonical Wnt activity in Xenopus embryos and in mammalian cells (16, 44, 46–48). Our data showing that an HFz6 mutant lacking CRD was incapable of repressing β-catenin-induced signaling suggested ligand-
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is reminiscent of the Drosophila Dfz3, which has been shown to serve as a natural attenuator of Wingless signaling during development (49). In addition, to Frizzleds that serve as Wnt receptors, other Frizzleds such as Fz6, the mouse Mfz3 and Mfz4, and the rat Rfz2 (13) as well as the Drosophila Dfz6 serve to down-regulate the canonical Wnt signaling pathway. The diversity of the Frizzled family of receptors resembles that of other families of Wnt regulators, such as the Frizzled-related protein (50) and the Dickkopf (51) families, which comprise both positive and negative regulators, serving to fine tune Wnt signaling whose tight regulation is essential for correct developmental processes. As TCF-β-catenin-DNA complex formation is regarded as a key step in Wnt signaling-induced tumorigenesis (18), its abrogation by HFz6 repressive signaling suggests HFz6 as a naturally occurring tumor suppressor gene whose silencing might be critical for tumor formation in humans.

Acknowledgments—We thank Dr. Stuart A. Aaronson of the Derald H. Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, NY, for critical reading of the manuscript. We are grateful to Drs. R. Agami, W. Birchmeier, J. Kitajewski, K. Matsumoto, T. Ishitani, R. G. Pestell, and C. Albanese and to B. Vogelstein for kindly providing various reagents used in this study.

REFERENCES

1. Peifer, M., and Polakis, P. (2000) Science 287, 1606–1609
2. Wodarz, A., and Nusse, R. (1998) Annu. Rev. Cell Dev. Biol. 14, 59–88
3. Miller, J. R., Hocking, A. M., Brown, J. D., and Moon, R. T. (1999) Oncogene 18, 7860–7872
4. Ishitani, T., Kishida, H., Hyodo-Miura, J., Ueno, N., Yasuda, J., Waterman, M., Shibuya, H., Moon, R. T., Ninomiya-Tsuji, J., and Matsumoto, K. (2003) Mol. Cell. Biol. 23, 131–139
5. Westfall, T. A., Brimeyer, R., Twedt, J., Gladon, J., Olberding, A., Furutani-Seiki, M., and Slusarski, D. C. (2003) J. Cell Biol. 162, 889–898
6. Pandur, P., Maurus, D., and Roth, M. (2002) Bioessays 24, 881–884
7. Pinson, K., Brennan, J., Monley, S., Avery, B. J., and Skarnes, W. C. (2000) Nature 407, 535–538
8. Tamaiz, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P., and Ho, X. (2000) Nature 407, 530–535
9. Wehrli, M., Doogan, S. T., Caldwell, K., O’Keefe, L., Schwartz, S., Vanael-Ohayon, D., Schejter, E., Tomlinson, A., and DiNardo, S. (2000) Nature 407, 527–530
10. Seidensticker, M. J., and Behrens, J. (2000) Biochim. Biophys. Acta 1495, 168–182
11. Behrens, J. (2000) Ann. N. Y. Acad. Sci. 910, 21–33
12. Polakis, P. (2001) Cell 105, 563–566
13. Kohli, M., Shields, L. C., Malbon, C. C., and Moon, R. T. (2000) J. Biol. Chem. 275, 12701–12711
14. van Es, J. H., Barker, N., and Clevers, H. (2003) Curr. Opin. Genet. Dev. 13, 26–33
15. Wedinger, G., and Moon, R. T. (2003) J. Cell Biol. 162, 753–755
16. Ishitani, T., Ninomiya-Tsuji, J., and Matsumoto, K. (2003) Mol. Cell. Biol. 23, 1379–1389
17. Ishitani, T., Ninomiya-Tsuji, J., Nagai, N., Nishita, M., Meneghini, M., Barker, N., Waterman, M., Bowerman, B., Clevers, H., Shibuya, H., and Matsumoto, K. (1999) Nature 399, 798–802
18. Polakis, P. (2002) Genes Dev. 16, 1857–1861
19. Gazit, A., Yaniv, A., Bafico, A., Pramila, T., Igarsashi, M., Kitajewski, J., and Aaronson, S. A. (1999) Oncogene 18, 5959–5966
20. Lorenzo, M. V., Long, J. E., Miki, T., and Aaronson, S. A. (1995) Oncogene 10, 2051–2055
21. Tokuhara, M., Hirai, M., Totani, Y., Terada, M., and Kato, M. (1998) Biochem. Biophys. Res. Commun. 243, 622–627
22. Bafico, A., Gazit, A., Pramila, T., Finch, P. W., Yaniv, A., and Aaronson, S. A. (1991) J. Biol. Chem. 267, 16180–16187
23. Bafico, A., Liu, G., Yaniv, A., Gazit, A., and Aaronson, S. A. (2001) Nat. Cell Biol. 3, 683–686
24. Kernise, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997) Science 275, 1784–1787
25. Chen, C., and Okumura, H. (1997) Mol. Cell. Biol. 17, 2745–2752
26. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
27. Elbashir, S. M., Harbort, J., Lendeckel, W., Tellen, A., Weber, K., and Tuschl, T. (2001) Nature 411, 494–498
28. Smalley, M. J., Sara, E., Paterson, H., Naylor, S., Cook, D., Jayatilleke, H., Fryer, L. G., Hutchinson, L., Fry, M. J., and Dale, T. C. (1999) EMBO J. 18, 2823–2835
29. Klein, P. S., and Melton, D. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8455–8459
30. Rogers, F. T., Hu, G., Dang, C. V., and Fearon, E. R. (1999) Mol. Cell. Biol. 19, 5696–5706
31. Muramatsu, S., Albert, I., Souza, B., Rubinfield, B., and Polakis, P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3046–3050
32. Ilyas, M., Tomlinson, I. P., Rowan, A., Pignatelli, M., and Bodmer, W. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10330–10334
33. Shultzman, M., Zhurinsky, J., Simcha, I., Albahane, C., D’Amico, M., Pestell, R.,
and Ben-Ze’ev, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5522–5527
34. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998) Science 281, 1509–1512
35. Nusse, R. (1999) Trends Genet. 15, 1–3
36. Rich, R. C., and Schulman, H. (1998) J. Biol. Chem. 273, 28424–284249
37. Umbhauer, M., Djiane, A., Geissler, C., Penzo-Mendez, A., Riou, J. F., Bourc'au, J. C., and Shi, D. L. (2000) EMBO J. 19, 4944–4954
38. Holmen, S. L., Salic, A., Zylstra, C. R., Kirschner, M. W., and Williams, B. O. (2002) J. Biol. Chem. 277, 34727–34735
39. Tolwinski, N. S., and Wieschaus, E. (2001) Development 128, 2107–2117
40. Bienz, M. (2002) Nat. Rev. Mol. Cell. Biol. 3, 328–338
41. Mulholland, D. J., Cheng, H., Reid, K., Rennie, P. S., and Nelson, C. C. (2002) J. Biol. Chem. 277, 17833–17843
42. Liu, J., Stevens, J., Rote, C. A., Yost, H. J., Hu, Y., Neufeld, K. L., White, R. L., and Matsunami, N. (2001) Mol. Cell 7, 927–936
43. Matsuzawa, S. I., and Reed, J. C. (2001) Mol. Cell 7, 915–926
44. Topol, L., Jiang, X., Choi, H., Garrett-Beal, L., Carolan, P. J., and Yang, Y. (2003) J. Cell Biol. 162, 899–908
45. Slusarski, D. C., Corcoran, V. G., and Moon, R. T. (1997) Nature 390, 410–413
46. Du, S. J., Purrel, S. M., Christian, J. L., McGrew, L. L., and Moon, R. T. (1995) Mol. Cell. Biol. 15, 2625–2634
47. Torres, M. A., Yang-Snyder, J. A., Purcell, S. M., DeMarais, A. A., McGrew, L. L., and Moon, R. T. (1996) J. Cell Biol. 133, 1123–1127
48. Olson, D. J., and Gibo, D. M. (1996) Exp. Cell Res. 241, 134–141
49. Sato, A., Kojima, T., Ui-Tei, K., Miyata, Y., and Saigo, R. (1999) Development 126, 4421–4430
50. Melkonyan, H. S., Chang, W. C., Shapiro, J. P., Mahadevappa, M., Fitzpatrick, P. A., Kiefer, M. C., Tomei, L. D., and Umanovsky, S. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13636–13641
51. Krupnik, V. E., Sharp, J. D., Jiang, C., Robison, K., Chickering, T. W., Ama-ravadi, L., Brown, D. E., Guyot, D., Mays, G., Leiby, K., Chang, B., Duong, T., Goodearl, A. D., Gearing, D. P., Sokol, S. Y., and McCarthy, S. A. (1999) Gene (Amst.) 238, 301–313
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J. Biol. Chem. 2004, 279:14879-14888.
doi: 10.1074/jbc.M306421200 originally published online January 27, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M306421200

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