Systematic mapping of WNT-Frizzled interactions reveals functional selectivity by distinct WNT-Frizzled pairs

Jacomijn. P. Dijksterhuis1,2, Bolormaa Baljinnyam2, Karen Stanger3, Hakki O Sercan2,a, Yun Ji2,b, Osler Andres2, Jeffrey S. Rubin2, Rami N. Hannoush3 and Gunnar Schulte1,4

1 Dept. Physiology & Pharmacology, Section of Receptor Biology & Signaling, Karolinska Institutet, Nanna Svartz Väg 2, S17177, Stockholm, Sweden
2 Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA
3 Department of Early Discovery Biochemistry, Genentech, 1 DNA Way, South San Francisco, California 94080, USA
4 Faculty of Science, Institute of Experimental Biology, Masaryk University, 611 37 Brno, Czech Republic

aCurrent address: Dokuz Eylul University, Faculty of Medicine, Department of Medical Biology, Izmir, Turkey
bCurrent address: Experimental Transplantation and Immunology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

* Running title: Receptor binding and functional selectivity of WNTs

To whom the correspondence should be addressed:
Gunnar Schulte, Section of Receptor Biology & Signaling, Dept. Physiology & Pharmacology, Karolinska Institutet, S-171 77 Stockholm, Sweden. Tel.: +46-8-52487933; Fax: +46-8-341280; E-mail: gunnar.schulte@ki.se
Rami N. Hannoush, Department of Early Discovery Biochemistry, Genentech, 1 DNA Way, South San Francisco, California 94080, United States. Tel.: +1-650-467-3696; E-mail: hannoush.rami@gene.com

Keywords: WNT pathway; WNT signaling; beta-catenin (β-catenin); disheveled; LDL receptor-related protein 6 (LRP6); myeloid cell; receptor; 32D cells; Frizzled; functional selectivity

Background: Wingless/Int-1/Frizzled (WNT/FZD) specificity and signaling outcome has not been systematically mapped so far.

Results: WNTs show selective binding to Frizzleds and respective WNT-FZD pairs exert functional selectivity in different downstream signaling pathways.

Conclusion: The WNT-FZD signaling system provides receptor-ligand selectivity.

Significance: Understanding WNT/FZD selectivity is crucial for development of WNT pathway inhibitors and further understanding of molecular interactions within the WNT receptor signaling complex.

ABSTRACT

The seven transmembrane spanning receptors of the Class Frizzled (FZD1-10) are bound and activated by the WNT family of lipoglycoproteins, thereby inducing a complex network of signaling pathways. Yet the specificity of interaction between mammalian WNT and FZD proteins and the subsequent signaling cascade downstream of the different WNT-FZD pairs has not been systematically addressed to date. In this report, we determine the binding affinities of various WNT proteins to different members of the FZD family by using biolayer interferometry and characterize
their functional selectivity in a cellular system. Using purified WNT proteins, we show that different FZD cysteine-rich domains (CRD) prefer to bind to distinct WNTs with fast on-rates and slow off-rates. In a 32D cell-based system engineered to overexpress FZD2, -4 or -5, we find that WNT-3A but not -4, -5A or -9B activates the WNT/β-catenin pathway through FZD2, 4, 5 as measured by phosphorylation of low-density-lipoprotein receptor-related protein 6 (LRP6) and β-catenin stabilization. Surprisingly, different WNT-FZD pairs show differential effects on phosphorylation of disheveled 2/3 (DVL2/3), revealing a previously unappreciated DVL isoform selectivity by different WNT-FZD pairs in 32D cells. In summary, we present extensive mapping of WNT/FZD CRD interactions complemented by analysis of WNT/FZD pair functionality in a unique cell system expressing individual FZD isoforms. Differential WNT-FZD binding and selective functional readouts suggest that endogenous WNT ligands evolved with an intrinsic natural bias towards different downstream signaling pathways, a phenomenon which could be of great importance in the design of FZD-target drugs.

The WNT/FZD signaling system has a prevalent role in physiology during adulthood. It also regulates a plethora of processes during embryonic development such as stem cell proliferation, migration and differentiation. Dysfunction of WNT/FZD signaling leads to impairment of cellular regulatory mechanisms, thereby leading to disease such as cancer, neurological and bone disorders, to name a few (1-3). Despite the physiological relevance of this pathway, little is known about the specificity of the interactions between the 19 members of the WNT family of lipoglycoproteins and their respective Frizzled cell surface receptors.

FZDs have the typical G protein-coupled receptor (GPCR) architecture of seven transmembrane segments and also show functional features of GPCRs (4-6). Therefore the FZDs together with the Hedgehog signal transducer smoothened were classified as an independent class within the superfamily of GPCRs by the IUPHAR (7) yet, unlike smoothened, little is known about the pharmacological features of the ten FZD isoforms (3). FZDs can act together with a range of single-membrane spanning co-receptors such as LRP5 and 6, receptor tyrosine kinase-like orphan receptor (ROR) 1 and 2, related to receptor tyrosine kinase (RYK) and proteoglycans, which serve as binding partners for WNTs and can thereby contribute to signal specification (8-10).

WNTs interact with FZDs through their cysteine-rich domain (CRD) at the N-terminus of the receptors (11,12), leading to initiation of distinct downstream signaling pathways. The WNT/β-catenin pathway is initiated by formation of a complex between WNT, FZD and LRP5/6, resulting in subsequent activation of the transcriptional regulator β-catenin. On the other hand, the mammalian β-catenin-independent pathways are transduced by WNT/FZD complexes and potentially other co-receptors, leading to complex signaling networks such as the WNT/Ca2+, WNT/RAC, WNT/RHO and a planar cell polarity (PCP)-like pathway (3,13,14). In all these different scenarios, the architecture of the WNT-receptor signaling complex may direct downstream signaling output, with the most proximal and central key players downstream of the WNT/receptor complex being the FZD-binding phosphoproteins disheveled 1, 2 and 3 (4,15).

While different signaling pathways initiated by WNTs have been characterized, the specificity of WNT-receptor interactions is still unclear. We showed earlier by using purified proteins that different WNT isoforms bind tightly to distinct regions on LRP6 (12), suggesting new possibilities for the molecular arrangement of WNT signaling complexes at the cell surface. Yet the specificity of the biochemical interactions between WNTs and FZDs remains elusive, and consequently the underlying mechanisms for selective activation of specific pathways by different isoforms of WNT/FZD complexes remain obscure. Drosophila WNTs were shown to exhibit distinct FZD-isofrom specificity shown by alkaline phosphatase-based interaction assays but the information for mammalian WNTs is very limited (16). An earlier study which utilized mouse, human and Drosophila FZD-CRDs showed that a Xenopus laevis XWNT-8/alkaline phosphatase fusion protein bound to mFZD5-CRD with 8 nM affinity (17). Also, previous data indicate that stimulation with different recombinant WNTs in N13 microglia-like cells expressing mRNA of several FZD isoforms resulted
in distinct signaling profiles depending on WNT/FZD pairings, suggesting signaling selectivity (18). Along the same lines, using fluorescence recovery after photobleaching experiments for the quantification of the mobile fraction of FZD\(_\text{CRD-Fc}\) expressed in HEK293 cells, it was shown that different recombinant WNTs differentially affect the lateral mobility of FZD\(_\text{CRD-GFP}\) (19), again suggesting ligand-receptor functional selectivity. To date, it has not been possible to measure WNT interaction with full length FZDs by classical quantitative receptor binding assays such as those utilizing radioactively-labelled WNT because (i) the labeling interferes with the WNT’s biological activity and (ii) there are no selective small molecule antagonists that enable distinction between specific vs non-specific binding at the receptor’s orthosteric site.

Here we determine the binding affinities of a subset of purified WNTs to different isoforms of soluble FZD CRDs in a cell-free system. Further, employing a murine bone marrow-derived 32D cell line, which expresses little or no FZD mRNA, combined with heterologous expression of specific FZD isoforms, we characterize the cellular effects of four different WNTs (WNT-3A, -4, -5A and -9B) in combination with either FZD\(_2\), FZD\(_4\) or FZD\(_5\). The observed functional selectivity between different WNT-FZD pairs suggests a model in which downstream WNT signaling output is determined by the identity of both WNTs and FZDs present at the cell surface.

**EXPERIMENTAL PROCEDURES**

**Materials** – Purified and carrier-free mouse and human WNT proteins were obtained from R&D Systems and used in the binding assays. Different isoforms of soluble mouse FZD CRD proteins fused to Fc were also commercially obtained (R&D Systems).

**WNT/ FZD CRD-Fc binding assays** – Binding kinetics were measured by BioLayer Interferometry (BLI) on an Octet Red instrument (ForteBio) as described earlier (12). Briefly, biosensors (anti-hIgG-Fc capture, AHC) were loaded with different recombinant FZD CRD-Fc proteins (R&D systems) in 50 mM Tris, pH 7.2, 300 mM NaCl, 5% (v/v) glycerol and 0.05% (w/v) Triton X-100. The loaded biosensors were washed in the same buffer before performing association and dissociation measurements with different purified WNT proteins for the indicated times. Kinetic parameters (\(k_{\text{on}}\) and \(k_{\text{off}}\)) and affinities (\(K_D\)) were determined from a non-linear fit of the data and the plots were generated using the Octet software. Each reported value represents the average of at least three experiments at different concentrations, with a fitted experimental curve for which the correlation coefficient (\(R^2\)) is above 0.98 and the maximal response signal for the association curve is above 0.07 nm. In the case of WNT-4, a weak response signal to FZD CRD-Fc domains was observed and the values derived from fitted experimental curves with maximal response above 0.03 nm and \(R^2 > 0.96\) were still considered in the calculation of kinetic parameters. The \(K_D\) represents the ratio between the \(k_{\text{off}}\) and \(k_{\text{on}}\), at equilibrium when the rate of complex formation by WNT and FZD CRD-Fc is equal to the rate of dissociation of the two components (see equation below) (20):

\[
K_D (\text{nM}) = \frac{k_{\text{off}}}{k_{\text{on}}} = \frac{[\text{WNT}][\text{FZD CRD-Fc}]}{[\text{WNT bound FZD CRD-Fc}]}
\]

Higher affinity binding pairs have a smaller \(K_D\) value, since less protein is required to establish equilibrium.

**Cell culture and stable transfection** - The IL-3-dependent mouse cell line 32D was maintained in RPMI 1640 medium, supplemented with 15% fetal bovine serum (FBS), penicillin (50 U/mL), streptomycin (50 μg/ml), L-Glutamine (2mM), 5% mouse myelomonocytic WEHI3B cell line conditioned medium and kept in a humidified atmosphere at 37°C and 5% CO\(_2\). WEHI3B conditioned medium containing IL-3 was collected from confluent monolayers, filtered and stored at -20°C. To obtain 32D cells expressing FZD\(_2\) or FZD\(_5\), 1x10\(^6\) cells were transfected with 2 μg DNA of HA-tagged FZD\(_2\) or HA-tagged FZD\(_5\) constructs, respectively, using Nucleofector 2b device (Lonza) according to the manufacturer’s protocol (Solution V, program E32). FZD\(_2\)-expressing 32D cells were created using Neon Transfection System (Life Technologies) according to the manufacturer’s protocol. Briefly, 2x10\(^5\) cells were mixed with 1 μg DNA of HA-FZD\(_5\) construct and electroporated in a 10 μl tip with two pulses having a width range of 30 ms and 1100V. Stable clones were selected with 3 mg/ml Hygromycin B (Calbiochem) and maintained.
in growth medium supplemented with 1mg/ml Hygromycin B.

RNA isolation, Quantitative Real Time-PCR and Reverse Transcriptase PCR - RNA was isolated from 32D cells (1x10^6 cells per isolation) using RNasey Mini kit (Qiagen, Hilden, Germany) and transcribed with the high-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed in triplicates with Taqman gene expression assay (Applied Biosystems) according to the manufacturer’s instructions. Measurements were done with ABI Prism 7000 sequence detector. Primer pairs used in this study were: FZD1 (Mm01320682_s1), FZD3 (Mm00445623_s1), FZD4 (Mm03053556_s1), FZD5 (Mm00445623_s1), FZD6 (Mm00433383_m1), FZD7 (Mm01255614_s1), FZD8 (Mm00434319_s1), FZD9 (Mm01206511_s1) and GAPDH (4352339E) as an internal reference (all from Applied Biosystems). Data are presented as ΔCt (C_{\text{FZD}} – C_{\text{GAPDH}}). Probe efficiency and primer validation were determined previously (21). Reverse Transcriptase PCR was performed as previously described (22,23), and PCR products were run on a 2.5% agarose gel with Gel Red Nucleic acid stain (Biotium). Primer specifications and T_{\text{anneal}} were reported previously (22).

PCR array profiling of 32D cells - For each cell line two biological replicates were used. RNA was isolated using RNasey kit and transcribed with RT² First-Stand cDNA Synthesis Kit following manufacturer’s protocol. QPCR was performed with the ready-to-use plate Mouse WNT signaling pathway PCR array from Qiagen (Cat#: 330231 PAMM-043A) according to the manufacturer’s protocol. QPCR was done on Mx3005P machine from Stratagene and data were analyzed through the web-based data analysis center from Qiagen.

Immunoblotting - Immunoblotting was performed as previously described (24). In short, after stimulation of serum deprived cells (2x10^5 per Eppendorf tube) with recombinant carrier-free WNT-3A, WNT-4, WNT-5A, WNT-9B (R&D systems) cells were centrifuged for 3 min, 3000 RPM at 4°C. Supernatant was aspirated and samples were lysed in 10% glycerol, 1% SDS, 100 mM Tris/HCl (pH 7.4), 1% β-mercaptoethanol and 0.01% bromophenol blue. Proteins were separated on a 10% SDS Polyacrylamide gel (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore). After blocking with 3% milk in Tris buffered saline with 0.05% Tween 20, membranes were incubated in the blocking buffer with the primary antibodies overnight at 4°C: mouse anti-β-catenin (1:1000, BD Bioscience #610153), mouse anti-DVL3 (1:500, Santa Cruz #sc-8027), rabbit anti-DVL2 (1:1000, Cellular Signaling #3216), rabbit anti-P-LRP6 (1:1000, Cell Signaling Technology #2568), mouse anti-HA tag (1:1000, Abcam #9110) and anti-mouse β-actin (1:30 000, Sigma #A5441). Proteins were visualized with appropriate horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse, Pierce) and the enhanced chemiluminescence method (Western-Lightening, PerkinElmer).

Data analysis - Statistical and graphical analysis was performed with Graph Pad Prism 5.0 software. Densitometry was quantified with the Image J software. Molecular weight (kD) of FZDs was determined by western blot and the related ImageLab software (Biorad). All data were analyzed with unpaired Student’s t test (*P < 0.05, **P<0.01, ***P<0.001). Immunoblotting data is shown as mean ±SEM and the binding data is presented as mean±SD. All experiments were repeated at least three independent times.

RESULTS

WNT/FZD CRD binding studies – To investigate the interaction between WNT and FZD, we developed a binding assay based on biolayer interferometry. Different isoforms of FZD (FZD1,2,4,5,7,8) CRD fused to Fc were loaded onto anti-hIgG biosensor tips. The sensor tip was washed on different assay and then transferred to different solutions containing a concentration range of purified recombinant WNTs (WNT-3A, -4, -5A, -5B, see Table 1, and Fig1A,B) which have been functionally validated in different assays in prior publications by us and others (6,12,18,19,21,22,24). We performed kinetic binding assays and determined the WNT-association rate k_{\text{on}} (1/M·sec), the WNT-dissociation rate k_{\text{off}} (1/sec) and the equilibrium dissociation constant K_D between different WNTs and FZD CRD’s. Representative binding curves of WNT-3A and WNT-5A binding to various FZD CRDs are shown in Fig 1A along with relative ranking of the K_D’s (Fig 1B). The k_{\text{eff}}, K_{\text{on}} and K_D
for the WNT and FZD CRD binding are shown in Table 1.

WNT-3A shows strong binding to FZD₄,₅,₇,₈ CRD (+++/, Kᵢᵢ = <10 nM, Table 1 and Fig 1A,B), suggestive of a tight complex between WNT-3A and these FZD isoforms on the cell surface. However, WNT-3A displays intermediate binding (+++, Kᵢᵢ = ~15 nM) to FZD₁,₂ CRD (Table 1 and Fig 1A,B). On the other hand, WNT-4, shows strong binding to FZD₈ CRD and very weak to intermediate binding to FZD₁,₂,₄,₅,₇ CRD (Table 1, Fig 1B). While both WNT-5A and -5B display strong binding to FZD₈ (+++/, Kᵢᵢ = 4-8 nM, Table 1, Fig 1A,B) and intermediate to strong binding to FZD₇ CRD while WNT-5B shows weak binding to these FZD CRD’s (Kᵢᵢ = >40 nM, Table 1, Fig 1B). Finally, WNT-7A, -9B, -10B, -11 and FZD₁₀ did not seem to show detectable binding to any of the chosen FZD CRD’s or WNTs in this study, respectively (data not shown).

Characterization of 32D cells – To assess the functional selectivity of WNT/FZD combinations, we chose the myeloid progenitor 32D cell line as a cell model because it expresses little or no mRNA for FZDs, as determined by qPCR (Fig 2A). As a control, mouse primary microglia cells express endogenous FZDs (Fig 2A) which are responsive to different WNT proteins (19,21,22,24,25). The lack of expression of endogenous FZDs in 32D cells (compare ΔCᵥ values of FZDs in 32D cells and microglia cells, Fig 2A) enables dissection of the function of a particular FZD via generating a stable 32D/FZDₓ-expressing cell line. To our knowledge, a cell system that expresses little or no endogenous FZDs has not been described previously. Moreover, we mapped the expression of other WNT co-receptors by RT-PCR (Fig 2B). LRP-5/6 but neither ROR1/2 nor RYK were expressed in 32D cells, thus providing the opportunity to activate the LRP5/6-dependent WNT/β-catenin pathway without involvement of other pathways, which are dependent on ROR1/2 or RYK signaling. In general, 32D cells appear to lack endogenous WNT-signaling components as shown in the PCR profiling provided in Fig 2C, even though that LRP5-6, DVL₁, 2, 3 are expressed and the β-catenin destruction complex machinery is functional.

We generated 32D cell lines, which stably express either FZD₂, FZD₄ or FZD₅. The FZD constructs contained two HA-tags which were engineered at the N terminus and downstream of the signal sequence to enable proper membrane targeting of the expressed protein and to allow assessment of FZD expression levels by immunoblotting (Fig 2C). The HA-FZD₂,₄,₅ signal was detected at a molecular weight of 53.1, 53 and 63.3 kD, respectively. These FZD constructs represent three of the four clusters of evolutionarily conserved Class FZD receptors (3). Since only a transient low level of FZD₃ and FZD₆ expression (representing the fourth cluster) was detected upon transfection of these constructs in 32D cells, we excluded these cell lines from our study.

β-catenin stabilization – The parental 32D cells were incubated with increasing concentration of purified soluble WNT-3A, -4, -5A and -9B for 2h (Fig 3A,B). The lack of observed β-catenin stabilization confirmed that the parental 32D cells were not responsive to WNT proteins in the absence of endogenous FZDs. However, expression of FZD₂, FZD₄ or FZD₅ in 32D cells resulted in a concentration-dependent increase in β-catenin protein levels in response to increasing concentration of WNT-3A (Fig 3A,B). On the other hand, maximal concentration of WNT-5A induced a slight stabilization of β-catenin in 32D/FZD₅ cells but not in 32D/FZD₂,₆, and neither WNT-4 nor WNT-9B induced β-catenin stabilization in any of the cell lines tested.

WNT-3A but not WNT-4, -5A or -9B induce phosphorylation of LRP6 - LRP6 serves as a coreceptor for FZDs and is central for the initiation and specification of the WNT/β-catenin pathway. The formation of a ternary WNT-FZD-LRP5/6 complex induces distinct LRP6 phosphorylation and recruitment to DVL-dependent signalosomes (26-30). Subsequently, this results in inhibition of glycogen synthase kinase 3-dependent β-catenin phosphorylation and degradation, thereby leading to β-catenin stabilization. In 32D/FZD₂, FZD₄ and FZD₅ cells, only WNT-3A induced phosphorylation of LRP6 (Fig 4A,B), consistent with the stabilization of β-catenin observed earlier (Fig 3A,B) and indicating that WNT-3A induces the β-catenin pathway via the aforementioned FZDs.
Differential phosphorylation of DVL2 and DVL3 - We used the same experimental setup as described above for β-catenin and P-LRP6 to investigate WNT-induced phosphorylation of DVL2 and DVL3 (as measured by electrophoretic mobility shift of phosphorylated DVL, known as PS-DVL). DVL is a key player in FZD signaling and mediates both β-catenin-dependent and β-catenin-independent pathways (24,25,31-35). Interestingly, parental 32D cells did not show a basal shift in DVL upon stimulation with WNT-3A, -4, -5A or 9B (Fig 5A,B and 6A,B), indicating lack of PS-DVL formation under these treatment conditions. However, expression of either FZD2,4,5 induced PS-DVL formation even in the absence of WNT stimulation. Moreover, stimulation of 32D/FZD2,4,5 cells with increasing concentrations of the four WNT proteins revealed differential effects on PS-DVL2 and -DVL3 in a manner that is dependent on the WNT-FZD pair present. PS-DVL2 formation was not observed in response to any of the WNTs in 32D/FZD4 cells, whereas PS-DVL3 formation occurred in FZD4-expressing cells upon stimulation with either WNT-4 or WNT-5A (Fig 5A,B and 6A,B). Additionally, WNT-3A and WNT-5A induced formation of PS-DVL3 but not PS-DVL2 in 32D/FZD3 cells. In contrast, WNT-4 induced both PS-DVL2 and PS-DVL3 in 32D/FZD3 cells. Finally, in 32D/FZD2 cells, WNT-3A, WNT-4 and WNT-5A caused the electrophoretic mobility shift of both DVL2 and DVL3.

DISCUSSION

In this study we investigated direct binding of different WNT proteins to various isoforms of FZD CRD and assessed the functional selectivity of the WNT-FZD pairs in initiating downstream signaling pathways. The general understanding of selectivity in WNT/FZD signaling has remained elusive, primarily due to the poor availability of purified active WNTs, which are challenging to obtain due to their hydrophobic lipid modification (34,36-38), as well as due to a lack of suitable, quantitative WNT-FZD binding assays. However, recent advances in protein expression and purification techniques have led to the development of a series of recombinant soluble WNTs (31,39,40) which have been validated by us and others for activity in different biological systems and functional assays (12,18,19,41). Yet, WNT-FZD binding has not been assessed systematically in a quantitative manner.

Our data from kinetic binding assays revealed new information about the behavior of WNTs and their binding to FZDs. Some WNTs seem promiscuous, such as WNT-3A which shows intermediate to strong binding to most of the FZD CRD’s examined in this study, whereas other WNT family members such as WNT-4, -5A and -5B seem more selective to different members of the FZD family. In the case of WNT-7A, -9B, -10B and -11, no binding to the FZD CRD domains was detected by biolayer interferometry assays. This result might be explained in light of a number of reasons: (a) these WNTs bind to other FZD CRD’s which were not among the panel of proteins examined in this study; (b) these WNTs require the full length of the FZD receptor to achieve high affinity binding; or (c) additional accessory proteins or yet poorly defined plasma membrane interactions could be required for establishment of functional high-affinity states. Nonetheless, for the other WNTs which show binding, the WNT/FZD CRD interaction seems to be the primary binding mode defining initial ligand receptor interaction, but additional contacts which span the extracellular surface loops and the FZD protein core might be involved, similar to what has been observed for other GPCRs binding peptide or protein ligands (42).

In order to assess the functional selectivity of WNTs towards individual FZDs, we chose 32D myeloid progenitor cells which exhibit little to no endogenous FZD expression, and generated versions of these cells, which stably overexpress individual representatives of Class Frizzled receptors. Of note is that these cells also express endogenous LRP5/6 proteins to levels sufficient to allow activation of WNT/FZD signaling. This unique cellular model enables the assessment of FZD pharmacology and can be potentially of use in drug screening campaigns. Unlike common mammalian cell lines, this cellular system offers for the first time the opportunity to assess the functionality of single FZD isoforms in a mammalian cell system. The parental 32D cells had low basal levels of β-catenin which did not change upon treatment with the different WNT proteins, emphasizing the notion that endogenous LRP5/6 expression in the absence of FZDs cannot initiate WNT-dependent β-catenin signaling. After heterologous expression of individual FZDs, the cells became responsive to...
WNTs as shown by WNT-3A-induced stabilization of β-catenin. Moreover, the parental 32D cells lacked a basal shift in DVL, i.e. the slower migrating band on DVL immunobLOTS was completely absent, and treatment of these cells with any of the chosen WNTs did not result in formation of PS-DVL, consistent with the lack of FZD expression in these cells. In addition, the lack of WNT-induced signaling in the parental 32D cells functionally supports our coreceptor expression profiling, arguing that even very low levels of ROR1/2 or RYK do not initiate WNT-mediated changes as measured in this study. In contrast, most mammalian cell lines express at least one or two FZDs at the mRNA level and show basal levels of PS-DVL (6,21,22,31,43), highlighting the basal signaling input in the presence of the receptors. In 32D cells, heterologous expression of either FZD2, FZD4 or FZD5 resulted in the formation of PS-DVL even in the absence of exogenous WNT stimulation. These findings indicate that expression of FZD is sufficient to induce phosphorylation of endogenous DVLs, consistent with earlier observations for FZD5 (44). Finally, WNT-3A, -4 and -5A demonstrated functional activity in the 32D/FZD cellular system as shown by β-catenin stabilization and P-LRP6 (WNT-3A) and PS-DVL2/3 (WNT-3A, -4 and -5A).

WNT-3A belongs to the group of WNTs, which generally induces WNT/β-catenin signaling and is an established ligand for FZD1,8 as was shown by immunoprecipitation, TOPflash and binding experiments (4,12,40). Our data suggest that WNT-3A forms a ternary complex with overexpressed FZD2,4,5 in the presence of endogenously expressed LRP5/6 to induce WNT/β-catenin signaling and P-LRP6 irrespective of the FZD isoform present in the cells. On the other hand, WNT-5A treatment led to a slight β-catenin increase in 32D/FZD5 only at a very high concentration (1000 ng/ml), consistent with an earlier report (45), but not in 32D/FZD2,4. Furthermore, neither WNT-4 nor WNT-9B induced β-catenin stabilization in 32D cells stably expressing FZD2, 4 or FZD5. Interestingly, WNT-9B has been previously shown to signal through FZD5 to induce luciferase activity in a TOPflash assay in HEK293 cells (38). One explanation for this discrepancy is that 32D cells are non-responsive to WNT-9B. We have previously shown that WNT-9B binds directly to LRP6 and that it induced WNT/β-catenin signaling in HEK293 and Hs578T cell lines (12,46). On the other hand, WNT-9B induced a β-catenin-independent PS-DVL formation, heterotrimeric G protein activation and cellular proliferation in N13 cells (18). These data indicate that WNT-9B, which does not show (a) binding to the FZD-CRDs tested here or (b) activity in 32D-FZD cells in the current study, can act on different pathways in cells with different receptor profiles. At this point, it is possible that WNT-9B can activate other FZDs that are not expressed in 32D cells or have not been tested in our binding assays.

WNT-4 is known to mediate β-catenin-independent signaling (47). However, when co-expressed with LRP6, WNT-4 has been shown to signal through FZD1 and FZD5 to induce TOPflash luciferase activity (45). The lack of WNT-4-induced β-catenin stabilization in FZD-overexpressing 32D cells might be due to differences in experimental conditions. In our studies, β-catenin stabilization is measured after 2 h of WNT stimulation, and is a more robust readout for WNT stimulation compared to TOPflash measurements, which are usually conducted after overnight treatment with WNTs. Long incubation times increase the possibility of cross talk with other pathways and indirect signaling loops.

A summary of the WNT/FZD binding and signaling results presented in this study is shown in Table 2. The observed binding of WNTs to FZD CRD seems to translate to functional activity in the 32D/FZD cell system. However, the affinity values for WNT-FZD binding do not seem to correlate with the extent of downstream signaling through activation of LRP6, DVL2/3 and β-catenin. For example, WNT-4 and WNT-5A display low affinity interaction with FZD2 and FZD5 respectively, yet they both show detectable effects on PS-DVL formation which are similar to the effects observed with other tighter binding WNTs. At this point, we can not rule out that other factors might be essential for the formation of a functionally high affinity ligand-receptor complex in living cells such as coreceptors, soluble co-factors and the requirement of full length FZD. Strikingly, the data summarized in Table 2 also imply a functional selectivity of WNT/FZD combinations with regard to differential phosphorylation of DVL isoforms. So far, only few clear functional differences in DVL isoforms have been discovered and systematically described. For example, the DVL knock-out phenotypes suggest distinct functional differences but also reveal a certain degree of redundancy (48). In our studies,
Receptor binding and functional selectivity of WNTs

WNT-3A/FZD₂ but not WNT-3A/FZD₄ induced formation of PS-DVL2/3, even though both WNT/FZD combinations induced β-catenin stabilization and phosphorylation of LRP6 (Figure 5-6 and Table 2). A similar trend was observed earlier in SN4741 neuronal precursor cells in which WNT-3A induced LRP6-dependent and PS-DVL-independent stabilization of β-catenin (24). Similarly, we showed recently that albeit a complete inhibition of β-catenin stabilization by Dickkopf-related protein 1 (DKK1), the WNT-3A-induced formation of PS-DVL is not blocked (33), thus uncoupling functional LRP6 signaling from the formation of PS-DVL. Even though WNT-3A did not induce DVL2/3 shift in 32D/FZD₄ cells, expression of FZD₄ on its own induced a basal PS-DVL compared to parental 32D cells. In contrast, WNT-4 and WNT-5A induced formation of PS-DVL without stimulating β-catenin stabilization and independently of the FZD type present (Fig 5-6 and Table 2).

For the WNT/FZD system, proper quantification of functional selectivity of WNT/FZD combinations in form of a bias factor (49), presents a challenge due to technical shortcomings such as unknown specific WNT activities and the lack of binding assays to full length receptors. Despite these pitfalls, the 32D/FZD cell system described here in combination with the biophysical WNT/FZD CRD binding assays serve as a good model system to pinpoint functional selectivity, identifying WNTs as ligands with natural bias at their receptors.

In summary, we systematically mapped WNT/FZD CRD binding of a set of soluble WNT and FZD CRD combinations. Further, we provide a link between direct binding and downstream signaling by systematic analysis of β-catenin stabilization and DVL activation for a selected subset of WNT/FZD combinations in a novel cell system comprising FZD-expressing 32D cells. The lack of endogenously expressed FZDs in these cells enabled for the first time the investigation of individual functional WNT/FZD pairs and their subsequent downstream signaling specificity. Our data showing preferential binding and signaling profiles of distinct WNT/FZD combinations suggest functional selectivity of WNTs. We conclude that WNT-3A, WNT-4 and WNT-5A are all functional binding partners to FZD₂,₄,₅ with appreciable affinity and putative natural bias for individual downstream signaling pathways. Further binding studies utilizing other soluble WNT proteins and full length FZDs are still needed to fully understand the selectivity of ligand-receptor interactions in the WNT system and its functional implications. Finally, other signaling readouts such as activity-based assays for heterotrimeric G proteins or second messenger measurements (50) could be included in the assessment of downstream mechanisms that specify WNT/FZD signaling in order to further investigate the pluridimensionality of WNT ligands and their Frizzled receptors.
REFERENCES

Receptor binding and functional selectivity of WNTs


ACKNOWLEDGEMENT

We would like to thank William Taylor and Eva Lindgren for excellent technical assistance.

FOOTNOTES

* The study was financially supported by grants from Karolinska Institutet, the Swedish Research Council (K2008-68P-20810-01-4, K2008-68X-20805-01-4, K2011-67X-20805-05-3), Swedish Cancer Society (CAN 2008/539, 2011/690), The KI-NIH Joint PhD program (JPD), Signhild Engkvist Foundations, Project KI-MU Grant CZ.1.07/2.3.00/20.0180. The work also was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute.

* The abbreviations used are: BLI; BioLayer Interferometry, CRD; cysteine-rich domain DKK1; Dickkopf-related protein 1, DVL; disheveled, FZD; Frizzled, GPCR; G protein-coupled receptor, LRP6; low-density-lipoprotein receptor-related protein 6, PCP; planar cell polarity, PS-DVL; phosphorylated and shifted Disheveled, ROR; receptor tyrosine kinase-like orphan receptor, RYK; Related to receptor tyrosine kinase, WNT; Wingless/Int-1.

FIGURE LEGENDS

FIGURE 1. Binding affinities for various WNTs binding to various FZD-CRDs, as measured by biolayer interferometry (A) Representative binding curves of WNT-3A and WNT-5A to FZD CRDs. (B) Summary of WNT/FZD CRD binding data. Tabulated are the binding values of four different WNT and various FZD CRD Fc proteins represented as: - no binding, + very weak binding (> 100 nM), ++ weak binding (40-100 nM), +++ intermediate binding (10-40 nM), ++++ strong binding (< 10 nM). NQ stand for weak binding, not quantifiable due to narrow response signal window in the binding curves. WNT-7A, -9B, -10B, -11 and FZD show no detectable binding to respectively any of the chosen FZD CRD’s or WNTs in this study (data not shown).

FIGURE 2. The myeloid progenitor cell line 32D expresses little or no endogenous FZDs but does express co-receptors LRP5/6. (A) The bar graph presents the FZD expression profile (FZD1-10) of 32D (hatched grey) and primary microglia cells (open bars) determined by quantitative PCR. n≥3, error bars show SEM. GAPDH was used as a housekeeping gene for normalization. By comparing FZD expression in primary microglia (previously published in Halleskog et al. (21)) and 32D cells, it becomes apparent that FZD levels in 32D cells are very low. (B) WNT co-receptor profile in 32D cells determined by reverse transcriptase PCR. LRP5, LRP6 but neither RYK, ROR1 nor ROR2 are expressed in 32D cells. Negative control consists of 32D cell cDNA prepared without reverse transcriptase; mouse tail genomic DNA or cDNA from GL261 cells (RYK) were used as positive control. (C) Table shows comparative PCR expression profiles of 32D and L929 cells including WNTs, FZDs, LRPs, DKKs and SFRPs. The table summarizes the average Ct values of two biological replicates. The following genes were not included on the PCR array: Wnt9b, Wnt10b, Fzd9, Fzd10, Ryk, Ror1 and Ror2. (D) Immunoblotting for HA-tagged FZDs in 32D cells expressing FZD2, FZD4 or FZD5. β-actin is used as loading control. The molecular weight (kD) of FZDs was determined with BioRad software.

FIGURE 3: WNT-3A evokes a dose-dependent β-catenin stabilization in mouse 32D/FZD2, FZD4 and FZD5 cells. (A) Bar graphs summarizes immunoblotting experiment quantifying β-catenin levels in lysates from parental mouse 32D, 32D/FZD2, 32D/FZD4 and 32D/FZD5 cells stimulated for 2 h with 0, 3, 10, 30, 100, 300 and 1000 ng/mL WNT-3A, -4, -5A or -9B. (B) Representative immunoblots showing β-catenin levels in response to increasing WNT concentrations. β-actin was used as loading control in all the experiments. Error bars provide standard SEM. *, P < 0.05; **, P < 0.01; N ≥ 3.
Receptor binding and functional selectivity of WNTs

FIGURE 4: WNT-3A induces dose-dependent phosphorylation of LRP6 in mouse 32D cells. (A) Bar graph shows phosphorylation of LRP6 (P-LRP6) in lysates from 32D/FZD2/FZD4/FZD5 cells stimulated for 2 h with PBS, 3, 10, 30, 100, 300 and 1000 ng/mL of WNT-3A. WNT-4, -5A, 9B did not evoke P-LRP6 in any of the 32D/FZD cell lines (not shown). (B) Representative immunoblots for the P-LRP6. β-actin was used as loading control in all the experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Densitometry data from N ≥ 3 independent experiments were used for graphical summary.

FIGURE 5: WNT-3A,-4,-5A induce dose-dependent phosphorylation and electrophoretic mobility shift of DVL2 (PS-DVL2) in mouse 32D/FZD cells. (A) Bar graph shows PS-DVL2 in lysates from 32D/FZD2/FZD4/FZD5 cells stimulated for 2 h with PBS, 3, 10, 30, 100, 300 and 1000 ng/mL of WNT-3A,-4,-5A. Densitometry data ratios, i.e. the quotient of the values from the upper (PS-DVL) and the lower DVL band, were normalized to the unstimulated control values. Basal PS-DVL2 was not detectable in the parental 32D cells (ND, not detectable). (B) Representative immunoblots showing the detection of PS-DVL2. The different forms of DVL2 are marked: DVL2: ◀; PS-DVL2: ◁. β-actin was used as loading control in all the experiments. Error bars provide standard SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001. N ≥ 3

FIGURE 6: WNT-3A,-4,-5A induce dose-dependent phosphorylation and shift of DVL3 (PS-DVL3) in mouse 32D/FZD cells. (A) Bar graph shows phosphorylation DVL3 (PS-DVL3) in lysates from 32D/FZD2/FZD4/FZD5 cells stimulated for 2 h with PBS, 3, 10, 30, 100, 300 and 1000 ng/mL of WNTs. Densitometry data ratios, i.e. the quotient of the values from the upper (PS-DVL) and the lower DVL band, were normalized to the unstimulated control values. Basal PS-DVL2 was not detectable in the parental 32D cells (ND, not detectable). (B) Representative immunoblots for detection of PS-DVL3. The different forms of DVL3 are marked: DVL3: ◀; PS-DVL3: ◁. β-actin was used as loading control in all the experiments. Error bars provide standard SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001. N ≥ 3

TABLES

TABLE 1. Binding affinities and kinetic constants for various WNTs binding to various FZD-CRDs, as measured by biolayer interferometry.

TABLE 2. Overview of the data presented in this study including WNT/FZD-CRD binding data and the functional response upon WNT/FZD binding.
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* NQ: weak binding, not quantifiable due to narrow response signal window in the binding curves.

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Dijksterhuis et al 2015 - Figure 1

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FZD₁ CRD

FZD₂ CRD

FZD₄ CRD

FZD₅ CRD

FZD₇ CRD

FZD₈ CRD

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Dijksterhuis et al. 2015 - Figure 2

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B

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D

E

F

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A

![Graph showing the expression levels of β-catenin as a percentage of control for different cell lines: 32D/parental, 32D/FZD₂, 32D/FZD₄, and 32D/FZD₅. The graph indicates the effects of WNT-3A, WNT-4, WNT-5A, and WNT-9B on β-catenin expression.]

B

![Blots showing the expression levels of β-catenin and β-actin for different cell lines: 32D/parental, 32D/FZD₂, 32D/FZD₄, and 32D/FZD₅. The blots are labeled with the corresponding WNT ligands: WNT-3A, WNT-4, WNT-5A, and WNT-9B.]

Dijkstraeus et al. 2015 - Figure 3
Dijkstra et al. 2015 - Figure 4

A

B

32D/FZD₂

P-LRP6
% of ctrl

32D/FZD₄

P-LRP6
% of ctrl

32D/FZD₅

P-LRP6
% of ctrl

WNT-3A

β-actin

P-LRP6

β-actin

% of ctrl
Dijksterhuis et al 2015 - Figure 5

A

32D/parental

ND ND ND ND ND

32D/FZD₂

32D/FZD₄

32D/FZD₅

PS-DVL2/DVL2 % of ctrl

WNT-3A WNT-4 WNT-5A WNT-9B

B

DVL2 β-actin

32D/parental

32D/FZD₂

32D/FZD₄

32D/FZD₅

WNT-3A WNT-4 WNT-5A WNT-9B

ND ND ND ND

% of ctrl

* ** *** ** *
Systematic Mapping of WNT-Frizzled Interactions Reveals Functional Selectivity by Distinct WNT-Frizzled Pairs

Jacomijn Petronella Dijksterhuis, Bolormaa Baljinnyam, Karen Stanger, Hakki Ogun Sercan, Yun Ji, Osler Andres, Jeffrey S. Rubin, Rami N. Hannoush and Gunnar Schulte

J. Biol. Chem. published online January 20, 2015

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