SHORT REPORT

Frizzled-4 C-terminus Distal to KTXXXW Motif is Essential for Normal Dishevelled Recruitment and Norrin-stimulated Activation of Lef/Tcf-dependent Transcriptional Activation

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The carboxy (C)-termini of G protein coupled receptors (GPCR) dictate essential functions. The KTXXXW motif C-terminus of Frizzleds (FZD) has been implicated in recruitment of Dishevelled (DVL). Through study of FZD4 and its associated ligand Norrin, we report that a minimum of three residues distal to the KTXXXW motif in the C-terminal tail of Frizzled-4 are essential for DVL recruitment and robust Lef/Tcf-dependent transcriptional activation in response to Norrin.

Keywords: Frizzled; Frizzled-4; carboxy-terminus; helix VIII; Dishevelled; Norrin

Background

The carboxy-terminal tail (C-tail) of GPCRs plays an essential role in receptor function and biology [1]. Mediating interactions with chaperones and downstream signaling elements, the C-tail may play a role in cell surface expression and downstream signal transduction [2]. Agonists binding to GPCRs have been shown to induce changes in C-tail conformation necessary for activating heterotrimeric G protein [3]. Prolonged agonist stimulation catalyzes phosphorylation of the C-tail, promoting arrestin binding, desensitization, and ultimately GPCR internalization [1].

Short amphipathic membrane interacting helix VIII at the transmembrane domain 7 (TM7) proximal end of the C-tail of GPCRs has been shown to be critical for G protein coupling and receptor trafficking [4, 5]. Although not reported for FZD, a crystal structure exists for the smoothened receptor [6]. To facilitate efforts in crystallization portions of the C-tail of GPCRs are often times truncated [7, 8]. For smoothened receptor, the flexibility of long unstructured regions of the C-tail required truncation at Q555, removing 200+ residues of the C-tail. The helix VIII was found to extend two residues beyond the tryptophan corresponding to that of FZD KTXXXW domains. Studies utilizing peptides encoding the C-tail of FZD suggest alpha-helicity and some role of the region immediately distal to KTXXXW in facilitating a receptor interaction with the PDZ domain of DVLs [9, 10].

Reports from studies employing full-length FZDs are not uniform concerning the function of the KTXXXW motif. Perturbation of the KTXXXW motif appears to inhibit the ligand-independent colocalization of Xenopus FZD3 with DVL [11]. Drosophila FZD2 lacking the KTXXXW motif, in sharp contrast, exhibited robust activation of Wnt/Wingless-induced Lef/Tcf-dependent transcription [12]. Promiscuity of various Wnts for Frizzleds occurs [13]. These possible promiscuous interactions are compounded at super physiological stoichiometry of FZD expressed in various cell lines [14]. These are formidable stumbling blocks to fine structure-activity analyses of FZD. To obviate these issues and probe the C-tail of FZD we took advantage of the FZD4-Norrin specificity [15, 16]. We found that the three residues QKC distal to the highly conserved KTXXXW domain of the FZD4 are required for substantial DVL recruitment and Lef/Tcf-dependent transcriptional activation.

Materials and Methods

Construction of plasmids

The mouse FZD4 construct containing the V5 tag between F37 and G38 in a prk5 vector (Addgene, Cambridge, MA) was used to generate mutants employed in this study.
The QuikChange Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) was employed with specific primers to mutate codons. To construct the mFZD3/C-tailmFZD3, mFZD4/C-tailmFZD4, and the mFZD1/C-tailmFZD1 chimeras in which the C-tail after the KTXXXW of mFZD4 was substituted with the corresponding region from mFZD1, mFZD4, or mFZD2, respectively, the overlap extension polymerase chase reaction (PCR) method was employed using the Phusion Hot Start II DNA polymerase (Thermo Scientific, Waltham, MA). The C-terminal green fluorescent protein (GFP)-tagged DVL2 construct was generated by inserting the human DVL2 gene into the pEGFPN vector (Clontech, Mountain View, CA). The constructs were verified by DNA sequencing.

**Cell culture**

Human embryonic kidney (HEK293) and HeLa cells (obtained from ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle's medium (Cellgro, Manassas, VA) supplemented with fetal bovine serum (10%, Hyclone, South Logan, UT), penicillin (100 μg/ml) and streptomycin (100 μg/ml, Corning, Manassas, VA) in a humidified atmosphere with a 5% CO₂ level at 37°C.

**Lef/Tcf-dependent transcriptional activation via luciferase reporter assays**

HEK293 cells were cultured in gelatin-coated 96 well plates (Greiner Bio-One, Frickenhausen, Germany) and then transfected at ~75% confluence with Lipofectamine 2000. Briefly, the conditions were as follows: 10 ng of GFP-tagged DVL2, 10 ng of Frizzled and empty vector to 250 ng per quadrant. Approximately 40 h after the transfections HeLa cells were then fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) at room temperature following the removal of the media. Fixed cells were then washed 3X with Hank’s Balanced Salt Solution (HBSS, Life Technologies, Carlsbad, CA). Following the third wash, the quadrants were incubated with two drops of Image iFX signal enhancer (Molecular Probes, Eugene, Oregon) and rocked for 30–60 min at room temperature. Cells were then washed with HBSS 3X prior to the addition of V5 antibody (Novex, Carlsbad, CA at 1:1000) diluted in 2% sterile filtered fraction V bovine serum albumin (BSA), (MP Biomedicals, Santa Ana, CA) containing HBSS solution overnight at 4°C. The following day the cells were washed 3X with HBSS and then incubated with Alexa Fluor 594 labeled secondary antibody (Molecular Probes, Eugene, Oregon) for 90 min at room temperature in the dark. Cells were washed again and maintained in HBSS at 4°C in the dark until Fluorescent and differential interference contrast (DIC) images were taken using a Fluoview FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) with a 60X oil immersion objective lens.

**Receptor surface expression measured by IFA**

Immunofluorescence assay (IFA) transfections were performed similarly to those of the functional assays, with 2 ng of receptor DNA per well. 24 h after, HEK293 cells were plated on gelatin-coated black plate clear bottom 96 well assay plates (Corning Inc., Corning, NY) in at least duplicate and fixed with 4% paraformaldehyde. Cells were subsequently washed with HBSS then blocked with 2% BSA. The cells were incubated with V5 antibody (Novex, Carlsbad, CA) at a 1:500 dilution, then washed with HBSS and incubated thereafter with Alexa Fluor 594 anti-mouse antibody diluted 1:1500. Cells were washed with HBSS and a SpectraMax M5 multimode plate reader (Molecular Devices, Sunnyvale, CA) was used to determine fluorescence readings for each well. Bar graphs display the % WT surface expression for each condition with the error bars representing the S.E.M. To determine the % WT surface expression for each condition the mean RFU value from the wells transfected without Frizzled plasmid was first subtracted from the other values. The mean of the resulting WT surface expression values was set as 100% WT surface expression.

**Data analysis**

To test for statistical significance between data, unpaired t-tests or one-way analysis of variance (ANOVA) followed by the Dunnett’s post test were conducted as described in the figure legends. p values < 0.05 established statistical significance.

**Results**

We analyzed the effects that truncation and substitution of the mouse Frizzled-4 C-tail (Figure 1) have on the ability of Norrin to induce Lef/Tcf-dependent transcriptional activation.
transcriptional activation (Figure 2A). Mutant mFZD4 (1-503) truncates the C-tail immediately beyond T503. Truncation at this position (mFZD4(1-503)) nearly abolished the ability of Norrin to activate Lef/Tcf-dependent transcription (Figure 2A). Restoring the wild-type sequence of the C-tail beyond T503 to N513 (mFZD4(1-503)) gradually ameliorated the loss of the Lef/Tcf-dependent transcriptional activation in response to Norrin to that of the WT response. We gauged the expression of mFZD4 and the truncations using amount of the surface expression (Figure 2B). All of the FZD4 constructs were expressed. Truncating the FZD4 carboxy-terminus at W504 to S508 resulted in some loss of cell surface expression. Although a positive correlation was observed between C-tail length and surface expression, the loss in activation was far greater than the apparent loss of some surface expression, suggesting that the simple reduction in cell surface mFZD4 could not account for the more profound loss of its function.

We probed the possibility that the changes in downstream signaling of the truncated mFZD4 might be accessible at the level of DVL (Figure 2C). We interrogated the ability of the mFZD4 and its variants to stabilize DVL at the cell surface. To enable these studies we made use of GFP-tagged DVL2, which is often times the most abundant DVL [17]. The ability of Frizzleds to stabilize DVL at the cell surface is essential to beta-catenin-dependent activation of the downstream pathway leading to Lef/Tcf-dependent transcriptional activation [11, 18]. Stabilization by FZD can be readily detected by confocal imaging of non-permeabilized cells expressing GFP-tagged DVL2 and V5-tagged mFZD4 (Figure 2C). We were unable to detect significant DVL2 recruitment and stabilization by mFZD4 C-tail mutants mFZD4(1-503), mFZD4(1-504), and mFZD4/C-tailmFZD4, or mFZD4/C-tailmFZD4 constructs differ from the mFZD4 WT construct. In these chimera, the region of the C-tail distal to the KTXXXW domain of mFZD4 has been substituted by the corresponding region of mFZD4 (mFZD4 residues R626 to V642), mFZD3 (mFZD3 residues A508 to A666 of mFZD3) or mFZD7 (mFZD7 residues R556 to V572), respectively. Note that for simplicity the mFZD4/mFZD3 C-tail is not shown in its entirety.
Figure 2: The FZD$_4$ C-tail modulates surface expression and receptor-DVL interaction. (A) V5mFZD$_4$ variants were overexpressed in HEK293 cells with hLRP5 and M50 reporter plasmid, stimulated with Norrin and Lef/Tcf-dependent luciferase activity was assayed as described. (B) Cell surface receptor expression detected by IFA in HEK293 cells transfected with the V5mFZD$_4$ variant shown and hLRP5. Following fixation the non-permeabilized cells were incubated with V5 primary antibody followed by incubation with a compatible Alexafluor594 secondary antibody and subsequent fluorescence quantification on a multimode plate reader. Statistically significant differences compared to WT as determined by an ANOVA analysis followed by the Dunnett’s post hoc test is indicated with an asterisk (*). (C) Confocal images of non-permeabilized HeLa cells co-transfected with GFP-tagged DVL2 and V5mFZD$_4$ WT or truncation variants using a V5 primary antibody.

Despite reports that under specific conditions FZD$_3$ can mediate beta-catenin-dependent signaling [11, 19] FZD$_3$ and FZD$_6$ have been reported to induce the least amount of Lef/Tcf-dependent activation amongst the 10 Frizzleds following the addition of various Wnts [13, 20]. With 159 amino acids following the KTXXXW
with a sequence highly homologous to the KTXXXW motif of the Frizzleds [6]. This domain has a short helix VIII parallel to the membrane encompassing the lysine extending to two residues beyond the tryptophan of the motif. A circular dichroism spectroscopy analysis of a peptide consisting of the FZD C-terminal in conjunction with molecular modeling suggests a helix VIII extending six residues beyond the KTXXXW motif of FZD [10]. In a membrane-mimicking environment a FZD C-terminal peptide exhibited a helix encompassing the leucine of the KTXXXW motif to 9 residues after the tryptophan [21]. Interestingly, the tryptophan residue, corresponding to W504 of mFZD, was shown to interact with the artificial lipid bilayer suggesting it could mimic the role of a C-terminal palmityl group which has been demonstrated to stabilize the helix 8 of various GPCRs [22, 23]. The mFZD_KC506-507AA mutation in which the only cysteine in the C-terminal was substituted with alanine exhibited normal Norrin-induced Lef/Tcf-dependent transcription signifying the absence or lack of a role in Lef/Tcf-dependent transcription of a palmitoylation site in the C-tail of FZD.

Other results obtained using peptides that map to the C-termini of Frizzleds suggest some function extending five to nine residues following the KTXXXW motif of FZD and FZD. Productive interaction between the FZD, with the DEP and PDZ domains of DVL appear to be influenced by this region of FZD [9, 24]. The present study indicates functionality that extends beyond the KTXXXW motif by more than three residues. The QC residues were shown to ameliorate receptor trafficking and Lef/Tcf-dependent transcription as a series of receptor constructs were generated including more of these residues. Although, WT-like signal transduction and trafficking observed with mFZD (1-513) was not detected with mFZD (1-507) signifying residues distal to QC may also have a role in enabling the formation of a helical structure critical for the life-cycle of the receptor.

Shortening the C-tail beyond C507 severely impaired normal DVL recruitment (as observed by FZD-DVL colocalization) and the ability of Norrin to activate Lef/Tcf-dependent transcription. The intracellular loops (loops) of Frizzleds also interact with DVL [18, 24]. It is possible that the Frizzled C-tail provides additional but essential interactions with DVL required for the receptor to participate in normal DVL recruitment and stabilization.

Multiple Frizzleds have been shown to mediate Wnt-induced activation of Lef/Tcf-dependent transcription to differing extents [20]. FZD and FZD for example, appear to activate Lef/Tcf-dependent transcription less robustly than FZD or FZD [13, 25]. The C-tails of FZD and of FZD are considerably longer than those of other Frizzleds. Substituting the corresponding C-tail of FZD distal to the KTXXXW sequence with that from FZD, FZD, or FZD, did not impact the ability of these mutant versions of FZD to mediate Norrin-induced Lef/Tcf-dependent transcriptional activation. The FZD, FZD, and FZD C-tails may provide

Discussion

In this study we show the C-tail of FZD, beyond the KTXXXW domain, affects several aspects of Frizzled-4 signaling and biology. The crystal structure of the smoothered receptor displays the region of the C-tail...
a structural role like that of FZD4's own native C-tail, *i.e.*, any secondary structure distal to transmembrane domain 7, such as helix VIII of FZD4, may form in the corresponding region of these other Frizzleds. The reduced ability of specific receptors to activate Lef/Tcf-dependent transcription may be due to factors outside of the ability of the C-tail to interact with DVL such as the potential of the receptor to synergize with LRPS/6 following the binding of a WNT ligand or other differences resulting from variations in the core of the receptors.

In summary, three residues distal to the KTXXXW motif of FZD4 are essential to normal FZD4-DVL interactions that are required for Lef/Tcf-dependent transcriptional activation by Norrin. Alpha-helicity in this region of the Frizzleds seems obligate for efficient protein-protein interaction with DVL and other downstream signaling elements.

**Conclusion**

This study demonstrates that substantial function of the FZD4 C-terminal tail minimally requires the three residues distal to the conserved KTXXXW domain. These additional residues QKC participate in cell-surface expression of Frizzled-4 and for signal propagation via Frizzled-DVL interactions that enable Norrin-dependent activation of Lef/Tcf-dependent transcription.

**Competing Interests**

The authors declare that they have no competing interests.

**Author's contributions**

AB and MP collected the data. AB wrote the draft manuscript. AB and MP generated the figures. HYW, CCM, and SG assisted in guiding the studies through collaborative discussions and edited the draft manuscript, final manuscript, and figures. Each author read and approved the submitted manuscript.

**Abbreviations**

ANOVA: analysis of variance; BSA: bovine serum albumin; C-tail: carboxy-terminal tail; C-termini: carboxy-termini; DIC: differential interference contrast; DVL: Dishevelled; FZD: Frizzled; GFP: green fluorescent protein; HBSS: Hank's Balanced Salt Solution; HEK293: human embryonic kidney; IFA: immunofluorescence assay; iloops: intracellular loops; M50: Super8xTOPFlash; mFZD: mouse Frizzled; PCR: polymerase chase reaction; RFU: relative fluorescence units; RLU: relative light units; S.E.M.: standard error of the mean; WT: wild-type

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