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WNT-5A stimulates the GDP/GTP exchange at pertussis toxin-sensitive heterotrimeric G proteins

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ABSTRACT

The lipoglycoproteins of the WNT family act on seven transmembrane-spanning Class Frizzled receptors. Here, we show that WNT-5A evokes a proliferative response in a mouse microglia-like cell line (N13), which is sensitive to pertussis toxin, thus implicating the involvement of heterotrimeric G proteins of the $G_{i/o}$ family. We continue to show that WNT-5A stimulation of N13 membranes and permeabilized cells evokes the exchange of GDP for GTP of pertussis toxin-sensitive G proteins employing [γ-^{35}S]GTP assay and activity state-specific antibodies to GTP-bound G_i proteins. Our functional analysis of the PTX-sensitivity of WNT-induced G protein activation and PCR analysis of G protein and FZD expression patterns suggest that WNT-5A stimulation leads to the activation of $G_{i2/3}$ proteins in N13 cells possibly mediated by FZD_5, the predominant FZD expressed. In summary, we provide for the first time molecular proof that WNT-5A stimulation results in the activation of heterotrimeric $G_{i2/3}$ proteins in mammalian cells with physiological protein stochiometry.

1. Introduction

Secreted lipoglycoproteins of the WNT family [1] orchestrate the embryonic development of multicellular organisms and tissue homeostasis. WNTs act as agonists of the Class Frizzled (FZD) receptors of unconventional seven transmembrane-spanning domain receptors, of which ten isoforms are known: FZD_{1-10} [2,3]. WNTs induce an elaborate network of signaling pathways [4,5] and dysfunctional WNT signaling leads to cancer and other diseases [6]. Historically, WNT signaling is divided into β-catenin-dependent and -independent signaling. Purified mammalian WNT-5A generally recruits β-catenin-independent signals [7] and activates mammalian FZD_2 [8], FZD_3 [9,10], FZD_4 [11], and FZD_5 [12].

Since the initial description of the primary sequence of the *frizzled* gene in *Drosophila melanogaster* it has been surmised that FZDs act as G-protein-coupled receptors (GPCRs) [13]. In fact, the Class FZD was recently defined as a novel class of GPCRs by the International Union of Clinical and Experimental Pharmacology [3,14] despite the lack of direct experimental evidence for a WNT- and FZD-mediated activation of heterotrimeric G proteins. Based on structural homologies of FZDs with classical GPCRs and loss-of-function data obtained using the $G_{i/o}$ protein-ribosylating toxin from *Bordetella pertussis* (pertussis toxin, PTX) as well as antisense- and siRNA-based approaches, heterotrimeric G proteins were implicated as participants in WNT/FZD signaling [for a recent review see Ref. 15]. Chimeric FZDs and genetic analysis of *D. melanogaster* also pointed in the direction of a requirement for G proteins in different branches of WNT/FZD signaling [16–20].

Heterotrimeric G proteins consist of a guanine-nucleotide-binding α subunit and the tightly associated βγ subunits. GPCRs act as guanine nucleotide exchange factors, which induce the release of GDP from the α subunit. GTP then binds to the nucleotide binding site, which results in the dissociation of the G protein from the receptor. Both α and βγ activate diverse effectors depending on the G protein family involved [21–23]. Despite compelling data on the G protein requirement for WNT/FZD signaling, it has so far been unclear whether WNTs can activate FZDs to induce the GDP/GTP exchange at heterotrimeric G proteins or if the FZD–G protein liaison might be indirect [2,15,24].

In the present study we provide direct evidence that WNT-5A stimulation leads to the activation of PTX-sensitive heterotrimeric G proteins in mammalian cells with natural receptor-G protein ratio.

2. Materials and methods

2.1. Cell culture

N13 and HEK293 cells were grown in DMEM, 10% FCS, 1% penicillin/streptomycin, and 1% L-glutamine (all from Invitrogen,

Abbreviations: FZD, Frizzled; GPCR, G protein-coupled receptor; [γS]GTP, Guanosine 5′-[γ-thio]triphosphate; SDS-PAGE, sodiumdodecylsulfate-polyacrylamide gelelectrophoresis; RT-PCR, reverse transcriptase polymerase chain reaction; QPCR, quantitative PCR.

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Paisley, UK). All cell plastic ware was from Sarstedt, Nümbrecht, Germany. Treatment with PTX (200 ng/ml, SIGMA) was done overnight. For measurement of proliferation N13 cells were seeded in 24-well plates (40 000/well), starved for 4 h and then stimulated for 24 h. Cells were then trypsinated and counted in a Bürker chamber (triplicates, n = 6). Saponin permeabilization was done on N13 cells in 24-well plates by 0.005% saponin in 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, and pH 7.4. After 5 min recombinant WNT-5A (R&D Systems, Abingdon, UK) was added. After an additional 10 min [γ-^{35}S] GTP (0.25 nM) and GDP (5 μM) were added. The reaction was stopped after 15 min by aspiration and cell lysis to prepare for analysis by native PAGE.

2.2. Membrane preparations

For bulk membrane preparation cells cultured in a 175 cm^2 flask were harvested using lifting buffer (PBS, 2 mM EDTA). The suspension was centrifuged and the cell pellet was resuspended in 20 mM HEPES, 10 mM EDTA, and pH7.4 and homogenized with a polytron homogenizer at 20 000 rpm on ice. Debris was separated by centrifugation at 1700 × g for 10 min. Next, the lysate was centrifuged at 40 000 × *g* for 1 h and the pellet was resuspended in 20 mM HEPES and 0.5 mM EDTA.

For small-scale membrane preparations N13 cells were grown in 60 mm dishes (1.2×10^6 cells/dish). Membranes were prepared with the ProteoJET membrane protein extraction kit (Fermentas AB, Stockholm, Sweden) according to the manufacturer's instructions.

2.3. [γ-^{35}S]GTP assay

Ten micrograms of membrane protein per well were diluted in 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, and pH 7.4 (final volume of 100 μl) in a 96-well Multiscreen HTS filter plate (Millipore, Billerica, MA, USA) and activated for 1 h at 37°C. Then GDP (5 μM, SIGMA), [γ-^{35}S]GTP (0.25 nM; 0.3 mCi; PerkinElmer) and recombinant WNT-5A (R&D Systems), alternatively 0.1% BSA in PBS as negative control, were added to a final volume of 300 μl. Plates were incubated at 30°C for 1 h, quickly aspirated, washed four times with ice-cold 0.9% NaCl and dried at 60°C overnight. Optiphase supermix scintillation liquid (30 μl/well; PerkinElmer Sverige AB, Upplands Väsby, Sweden) was added and radioactivity was quantified on a Wallac Trilux 1450 MicroBeta.

2.4. HEK293 cell transfection

HEK293 cells were seeded into 24-well plates and transfected using the $CaPO_4$ method. Expression plasmids for the various constitutively active QL mutants of Gα subunits were from the Missouri S&T cDNA Resource Center. Analysis was performed 24 h posttransfection.

2.5. PAGE and immunoblotting

For native polyacrylamide gelelectrophoresis (PAGE) cells were lysed on ice in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF and were homogenized with a 30 G needle. Debris was removed by 10 min centrifugation at 14 000 × *g*. Protein concentration of the supernatant was determined and 50 μg/lane was mixed with 5× loading buffer (50% glycerol, 0.5 M Tris/HCl, pH 7.4) and analyzed by 12% PAGE without sodiumdodecylsulfate. For SDS-PAGE on membrane preparations N13 membranes were solubilized in 50 mM Tris/HCl, pH 7.4, Na-deoxycholate 0.25%, NaCl 150 mM, EDTA 1 mM, NaF 1 mM, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA), and 1% Triton X-100, denatured in 100 mM Tris/HCl pH7.6, 10% SDS, 20% β-mercaptoethanol, 15% glycerol, and bromophenol blue and analyzed on a 12% SDS-PAGE. Proteins were electrotransferred to the PVDF membrane. The primary antibodies used were: mouse anti-active G_i (G_i-GTP) antibody (1:1000; cat# 26901; NewEast Biosciences, Malvern, PA, USA), rabbit anti-FZD_5 (1:1000; cat# 06-756; Millipore), goat anti-FZD_5 (1:1000; cat#AF1617; R&D Systems), and rabbit anti $G\alpha_{i3}$ (1:1000; cat#: sc-262; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). HRP-coupled secondary antibody in combination with the Western Lightning ECL detection kit (PerkinElmer) was used.

2.6. PCR

RNA was isolated from N13 cells using the RNeasy Mini Kit (Quiagen, Hilden, Germany). cDNA was transcribed with the high-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). PCR was performed as reported previously [25] and PCR products were run on 2.5% agarose/ethidiumbromide gels. For primer sequences see Table S1. Quantitative PCR was done with the TaqMan gene expression assay (Applied Biosystem). Primer pairs FZD_1 (Mn01320682_s1), FZD_2 (Mn02524776_s1), FZD_4 (Mn03053556_s1), FZD_5 (Mn00445623_s1), FZD_7 (Mn01255614_s1), FZD_8 (Mn00433419_s1), and FZD_9 (Mn01206511_s1) were from Applied Biosystems. Reactions were performed using an ABI Prism 7000 sequence detector (Applied Biosystems) and data are presented as the difference in cycle number to reach the detection threshold using 18 S rRNA as internal reference ($\Delta C_t = C_{tFZD} - C_{t18S}$).

2.7. Statistical analysis

Graph Pad Prism 5 was used for graphical and statistical analysis. Data were analyzed by unpaired, two-tailed *t*-test (Fig. 2A) or 1-way ANOVA/Newman–Keuls multiple comparison posttest (Figs. 1, 4, 5B). All experiments were repeated at least three times.

3. Results and discussion

3.1. WNT-5A-mediated N13 proliferation is PTX sensitive

Many experimental studies have indicated a role of heterotrimeric G proteins for different branches of WNT signaling [3], but it is still unclear whether WNTs can activate these G proteins in mammalian cells at physiological receptor-G protein ratios. To address this question we first investigated the effect of PTX on the WNT-5A-dependent proliferation in microglia cells. Proliferation of these immunocompetent cells of the nervous system is an intrinsic feature

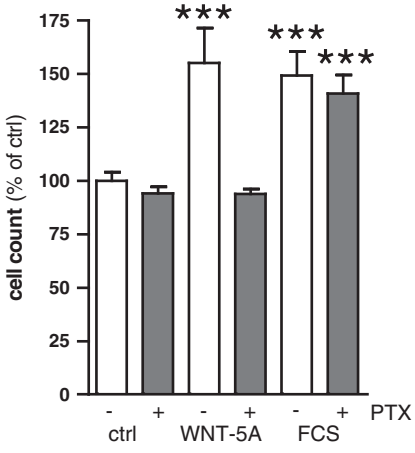

Fig. 1. WNT-5A-induced proliferation of N13 microglia-like cells is sensitive to pertussis toxin (PTX). Serum-deprived N13 cells were stimulated with WNT-5A or fetal calf serum (10% FCS) in the presence or absence of 200 ng/ml PTX for 24 h. Cells were counted in triplicates ($n = 3$) in a Bürker chamber and the response was normalized to control (ctrl) stimulation in the absence of PTX. ***$p < 0.001$, 1-way ANOVA, Newman–Keuls multiple comparison test.