VIEW POINTS

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WNT Unrelated Activities in Commercially Available Preparations of Recombinant WNT3a

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ABSTRACT

WNT signaling pathways play an important role in both development and disease. By analyzing the signaling capabilities of commercially available WNT3a preparations towards the PI3K/AKT/GSK3 signaling pathway, we discovered unexpected inconsistencies from lot to lot of recombinant WNT3a. We provide evidence that: (1) The ability to trigger AKT/GSK3 signaling varies dramatically between different lots of WNT3a, without any variation in their ability to activate the canonical WNT/β-catenin signaling. (2) sFRP1, a WNT signaling inhibitor, is unable to interfere with the activation of AKT/GSK3 signaling induced by some of the WNT3a lots. (3) Pharmacological inhibition of AKT/GSK3 phosphorylation by PI3K inhibitors fails to affect the stabilization of β-catenin, the central effector of the canonical WNT/β-catenin signaling pathway. In summary, while all tested lots of recombinant WNT3a activated WNT/β-catenin pathway, our results suggest that individual lots of recombinant WNT3a activate the PI3K/AKT/GSK3 pathway in a WNT-independent manner, hampering thus the analysis of regulation of PI3K/AKT/GSK3 by WNT ligand. J. Cell. Biochem. 111: 1077–1079, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: WNT PATHWAY; LIGAND; PURIFICATION; SIGNALING; AKT/GSK3 PHOSPHORYLATION; SFRP1; BETA-CATENIN

Signaling by the WNT family of glycolipoproteins plays critical roles in a growing list of biological systems, where it regulates different aspects of embryo development, tissue homeostasis and disease, including cancer [MacDonald et al., 2009; van Amerongen and Nusse, 2009]. Similarly, the number of described signaling components implicated in WNT signaling pathways grows continuously. One such component is AKT/PKB kinase, which was proposed as being a downstream effector of WNT signaling based on experiments utilizing either overexpression [Fukumoto et al., 2001] or treatment with partially purified [Constantinou et al., 2008] or commercially available WNT ligand [Almeida et al., 2005; Kim et al., 2007]. Importantly, GSK3, a downstream target of AKT, is a well established and critical component of the WNT/β-catenin pathway [MacDonald et al., 2009]. However, genetic evidence for the role of AKT kinase in WNT signaling is missing and the cellular consequences of AKT/GSK phosphorylation in the WNT/β-catenin pathway are far from understood.

We thus decided to directly address if WNT ligand has the ability to trigger the AKT/GSK3 signaling. We stimulated various cell lines (SN4741, COS7, HEK293A) by commercially available recombinant mouse WNT3a (1324-WN, R&D Systems). WNT3a is a "canonical" WNT ligand, which upon interaction with its surface receptors, Frizzled and LRP5/6, triggers WNT/β-catenin signaling. This signaling pathway is mediated by stabilization and accumulation of β-catenin in the cytosol, translocation of β-catenin into the cell nucleus, interaction with TCF/LEF and subsequent activation of target genes. Strikingly, we discovered an inconsistency in the ability of different lots of WNT3a to induce AKT and/or GSK3 phosphorylation. Stimulation of SN4741, COS7 and HEK293a cells with a lot that we subsequently termed "High AKT signaling" (HTR196051) led to robust phosphorylation of AKT (Ser473) and its target GSK3 (Ser9) (Fig. 1A and data not shown). Another lot (HTR340871), termed "Low AKT signaling," showed only subtle ability to trigger AKT and GSK3 phosphorylation. However, three other lots of WNT3a (HTR4209031, HTR3408071, HTR5809091) completely lacked the capability to trigger phosphorylation of AKT and/or GSK3 (referred to as "No AKT signaling" lots, Fig. 1A). In these experiments, EGF was used as positive control for AKT phosphorylation, confirming thus that the lack of activity was not due to inability of SN4741 cells to respond to AKT activating stimuli

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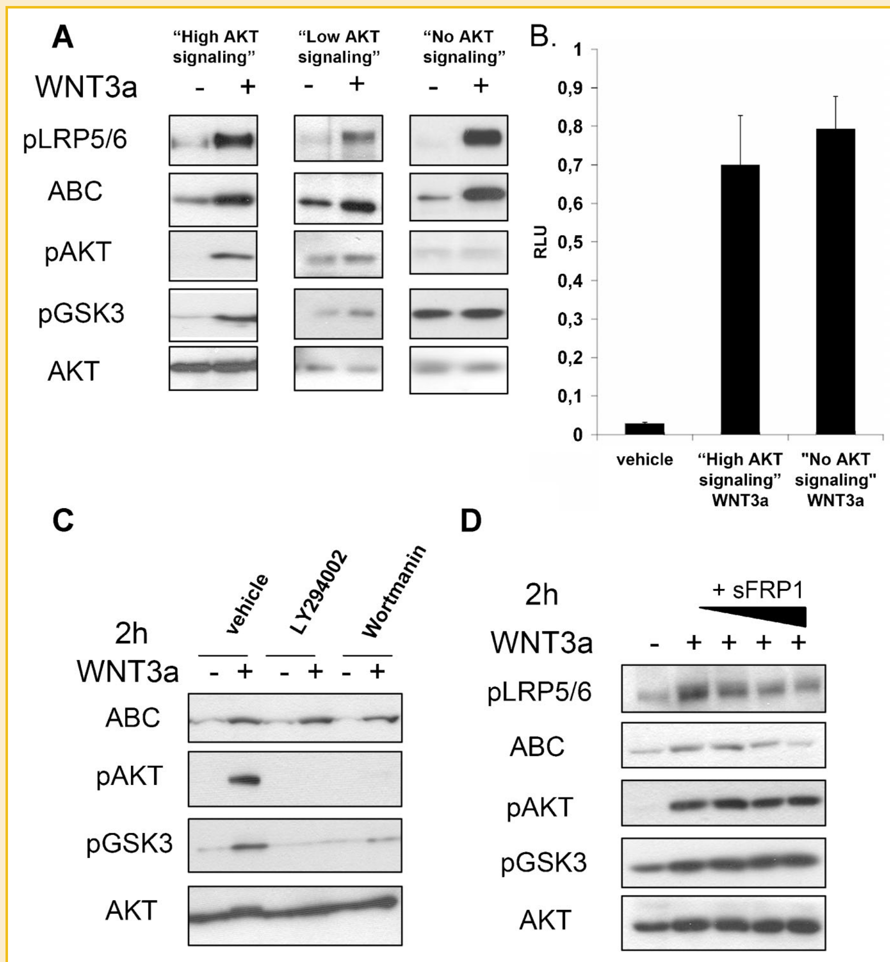

Fig. 1. A: SN4741 cells were stimulated with various lots of WNT3a; activation of downstream signaling was assessed by Western blotting. Note that all tested lots of WNT3a induce the two best defined protein readouts of WNT pathway activation—phoshorylation of the Wnt co-receptor LRP5/6 (Ser1493/Ser1490) and increase in the active β-catenin (ABC), but they differ in their ability to activate AKT (Ser473)/GSK3 (Ser9) phosphorylation. Blots were re-probed for total AKT to confirm equal amount of protein between control and WNT3a-stimulated condition for each lot of WNT3a tested. B: SN4741 cells were co-transfected with Super-TOPFlash (reporter for Wnt/β-catenin signaling-induced transcription) and Renilla luciferase (internal control) reporters and stimulated with WNT3a for 24 h. Cell lysates were analyzed using Dual luciferase assay (Promega). No difference in reporter activation between "High AKT signaling" WNT3a and "No AKT signaling" WNT3a was observed. RLU-relative luminescence units show signal from Super-TOPFlash luciferase normalized to Renilla luciferase and expressed as mean ± SD. C: SN4741 cells were treated with PI3K inhibitors, 10 μM LY294002 or 50 μM Wortmanin, for 30 min before stimulation with WNT3a. While WNT3a-induced AKT/GSK3 phosphorylation was blocked, the ability to induce increase in ABC was not affected, as assessed by Western blotting. D: SN4741 cells were treated with increasing dose of sFRP1 (inhibitor of WNT signaling) 5 min before stimulation with WNT3a. Western blotting analyses revealed that treatment with sFRP1 dose-dependently abrogates WNT3a-induced ABC accumulation and LRP5/6 phosphorylation, but fails to interfere with WNT3a-induced AKT/GSK3 phosphorylation. The antibodies used for WB were: ABC/8E7 (Millipore), pLRP5/6, pAKT, pGSK3, AKT, and GSK3 (Cell Signaling Technology).

(not shown). Interestingly, none of the tested lots of recombinant WNT3a significantly differed in their ability to activate WNT/β-catenin signaling, as demonstrated by activation (dephosphorylation) of β-catenin (ABC) and phosphorylation of LRP5/6 (Fig. 1A). In addition, we found no quantitative difference in the activation of the WNT/β-catenin pathway between the "High AKT signaling" lot and lot of WNT3a from the "No AKT signaling" group, as measured by TOPFlash luciferase reporter assay (Fig. 1B).

The high variability between WNT3a lots at inducing AKT and/or GSK3 phosphorylation, and the unusual signaling properties of the "High AKT signaling" lot, prompted us to investigate the signaling mechanism of this particular lot of WNT3a in more detail. This lot was able to induce AKT phosphorylation very rapidly, 2–3 min after stimulation (data not shown). PI3K has been proposed as an upstream regulator of WNT3a-induced AKT/GSK phosphorylation [Sonderegger et al., 2010]. Indeed, use of the PI3K inhibitors Wortmanin and LY 294002 abolished the induction of phosphorylation of AKT and GSK3 by the "High AKT signaling" WNT3a (Fig. 1C). However, the complete block of AKT activation by PI3K inhibitors did not alter the ability of the "High AKT signaling" lot of WNT3a to induce accumulation of ABC (Fig. 1C). These findings suggest that the phosphorylation of AKT/GSK is not required for WNT3a-induced β-catenin stabilization in SN4741 cells. Finally, we examined whether the effects of "High AKT signaling" WNT3a on AKT/GSK could be blocked by inhibition of WNT signaling. We thus used SFRP1, a soluble inhibitor of WNT signaling that binds WNT

ligands extracellularly and has previously been described to block WNT-mediated AKT activation [Fukumoto et al., 2001]. SFRP1 (1384-SF, R&D Systems) dose dependently blocked the activation of the WNT/β-catenin pathway by the "High AKT signaling" WNT3a lot, but did not attenuate phoshorylation of AKT and/or GSK3 (Fig. 1D).

In summary, our analysis of the signaling pathways activated by different lots of recombinant WNT3a led us to the following conclusions: (1) The capacity of different WNT3a lots to induce phosphorylation of AKT/GSK3 varies dramatically while they all activate WNT/β-catenin signaling. (2) PI3K inhibitors prevented the induction of AKT/GSK phosphorylation by certain WNT3a lots but failed to affect the stabilization of β-catenin, the crucial effector of WNT/β-catenin pathway. (3) sFRP1, a WNT signaling inhibitor, was unable to block AKT/GSK3 phosphorylation. All these findings suggest that individual lots of recombinant WNT3a differ in their activity. While all of them activate WNT/β-catenin signaling, only few activate PI3K/AKT/GSK3 signaling. Importantly, our results indicate that activation of the PI3K/AKT/GSK3 pathway by "recombinant WNT3a" is not dependent on bona fide WNT signaling or WNT ligand itself.

Despite these limitations, it should be noted that the purification of recombinant WNT proteins [Willert et al., 2003; Schulte et al., 2005; Sousa et al., 2010] has represented a major breakthrough in the field as it has allowed pharmacological approaches to study WNT signal transduction. The preparations of commercially available mouse recombinant WNT3a protein used in this study contained 75% of correctly folded full length WNT3a protein and 25% of missfolded and/or proteolytically cleaved WNT3a (personal communication with R&D Systems). It remains to be determined whether missfolded WNT3a or different proteolytic products may contribute to the observed discrepancies in signaling capabilities and whether there are other unknown factors responsible for the different activities in the preparations. Importantly, our findings shed light on some inconsistencies in the literature and underscore the need of careful interpretation of experiments utilizing commercially available recombinant WNT ligands.

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